Effects of amyloid β1-42 investigated by *in vivo* single-unit electrophysiology: Ionotropic glutamate receptors and neuroprotection

Ph.D. Thesis

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Science never solves a problem without creating ten more

George Bernard Shaw

List of Publications

Full papers, directly related to the subject of the thesis

I. Szegedi V, Fulop L, Farkas T, Rozsa E, Robotka H, Kis Z, Penke Z, Horvath S, Molnar Z, Datki Z, Soos K, Toldi J, Budai D, Zarandi M, Penke B.: *Pentapeptides derived from Abeta1-42 protect neurons from the modulatory effect of Abeta fibrils--an in vitro and in vivo electrophysiological study.* Neurobiol Dis. 2005 Apr;18(3):499-508.

II. Viktor Szegedi, Gábor Juhász, Dénes Budai, Botond Penke: *Divergent effects of Aβ1-42 on ionotropic glutamate receptor-mediated responses in CA1 neurons in vivo.* Brain Res. 2005 Nov 16;1062(1-2):120-6

III. Viktor Szegedi, Gábor Juhász, Éva Rózsa, Gabriella Juhász-Vedres, Zsolt Datki, Lívia Fülöp, Zsolt Bozsó, Andrea Lakatos, Ilona Laczkó, Tamás Farkas, Zsolt Kis, Géza Tóth, Katalin Soós, Márta Zarándi, Dénes Budai, József Toldi, Botond Penke: *Endomorphin-2, an endogenous tetrapeptide protects against Aβ1-42 in vitro and in vivo.* FASEB J. 2006 Apr 24

Patents directly related to the subject of the thesis

I. B. Penke, M. Zarándi, G. Tóth, Z. Datki, L. Fülöp, V. Szegedi, Z. Molnár, K. Soós, T. Farkas, Z. Penke, Y. Verdier, T. Janáky, G. Tóth, Peptides and peptidomimetics for the therapeutical treatment of neurodegenerative diseases associated with abnormal protein folding to amyloid-like deposits, Preliminary patent, submitted in March, 2005

Full papers, not related to the subject of the thesis

I. Molnár, Zs.; Budai D.; Soós, K.; Lengyel, I.; Penke, B.; Szegedi, V.: *Enhancement of NMDA responses by ß-amyloid peptides in the hippocampus in vivo.* Neuroreport. 2004 Jul 19;15(10):1649-52

II. Viktor Szegedi, György Bárdos, László Détári, Attila Tóth, Ilona Banczerowski-Pelyhe, Ildikó Világi: *Transient alterations in neuronal and behavioral activityfollowing bensultap and fipronil treatment in rats.* Toxicology, 2005 Oct 15;214(1-2):67-76.

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Abbreviations

AAI	Amyloid aggregation inhibitor
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
AICD	AβPP intracellular domain
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole propionic acid
ANOVA	Analysis of variance
Aph-1	Anterior pharynx-defective phenotype
APLP	AβPP like proteins
Aβ	β-amyloid peptides
AβPP	Amyloid-β precursor protein
BACE-1	β-site AβPP-cleaving enzyme
BSB	β-sheet breaker
CA1	Cornu Ammonis 1
CNS	Central nervous system
COX2	Cyclooxygenase-2
CTF	COOH-terminal fragment of Aβ
DAMGO	D-Ala^2, N-Me-Phe^4, Gly^5-ol)-enkephalin
EEAT	Excitatory amino acid carrier
EEC	European Communities Council
End-2	Endomorphin-2
FAD	Familial Alzheimer's disease
FDA	Food and Drug Administration
fEPSP	Field excitatory postsynaptic potential
FRGDS	Phe-Arg-Gly-Asp-Ser
FRHDS	Phe-Arg-His-Asp-Ser
GABA	γ-aminobutyric acid
GGGGG	Pentaglycin
Glu	Glutamate
GVVIAa	Gly-Val-Val-Ile-Ala amide
i.p.	Intraperitoneal
IDX	4'deoxy-4'iodorubicin
IIGLM	Ile- Ile-Gly-Leu-Met
IL-1β, IL-6	Interleukin-1β, interleukin-6
JNK	C-jun N-terminal kinases
KA	Kainic acid
KLVFF	Lys-Leu-Val-Phe-Phe
KLVFFA	Lys-Leu-Val-Phe-Phe-Ala
KPI	Kunitz-type serine protease inhibitor
LFA-1	Lymphocyte function-associated antigen–1
LPFFD	Leu-Pro-Phe-Phe-Asp
LPYFDa	Leu-Pro-Tyr-Phe-Asp amide
LRP	Low density lipoprotein receptor-related protein
LTP	Long term potentiation
LVFFA	Leu-Val-Phe-Phe-Ala
mAβ	Monoclonal antibody against Aβ
mGluR	Metabotropic glutamate receptor

MIP-1α	Macrophage inflammatory protein-1α
MOR	Mu-opioid receptor
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFT	Neurofibrillary tangles
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drugs
Pen-2	Presenilin enhancer
PHF	Paired helical fragment
Pr- IIGLa	Propionyl-Ile-Ile-Gly-Leu amide
PS1	Presenilin 1
PSGL	P-selectin glycoprotein ligand-1
RHDS	Arg-His-Asp-Ser
RIIGL	Arg-Ile-Ile-Gly-Leu
RVVIA	Arg-Val-Val-Ile-Ala
SEM	Standard error of means
SP	Senile plaques
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β
TM	Transmembrane domain
TNF-α	Tumor necrosis factor α
YPFF	Tyr-Pro-Phe-Phe
βAPPs	Secreted soluble ectodomain of AβPP
τ	Tau protein

General Introduction

The leading cause of senile dementia among the elderly is Alzheimer's disease (AD). Nearly 3% of people aged 65 show signs of the disease, while 25-50% of people aged 85 have symptoms of this progressive mental deterioration, and estimates place the number of affected individuals to 29 million by 2020. The incidence of AD increases exponentially with age and there is no leveling off in very old age (at least up to the age of 90) (Jorm and Jolley, 1998). If mild cases are included, the prevalence is as high as 10.3 % in a western population over 65 years of age and increases to almost 47 % for people over 85 years of age. In the 85+ group, the disease is most prominent in females (ratio 2.8/1 over 75 years of age) (Forsyth and Ritzline, 1998). Although by 2050 the number of people affected with AD will be doubled, an effective treatment for the disease is still lacking.

In 1907, Alois Alzheimer was the first to describe a form of dementia in a 55-year old female of which *post mortem* staining of the brain – with at that time newly available silver stains – revealed the presence of tangled fibres and clusters of degenerating nerve endings (Katzman, 1986). Furthermore, Alzheimer described progressive memory impairment, disordered cognitive functions, altered behavior (paranoia, delusions) and decline in language function, all features that are still identifiable in Alzheimer patients today (Selkoe, 2001). This collection of symptoms came to bear his name and is nowadays still a topic of extensive research. The histological identifiable hallmarks of AD are: 1) intraneuronal, cytoplasmatic deposits of neurofibrillary tangles (NFT), 2) extracellular amyloid deposits called neuritic plaques, 3) cerebrovascular amyloidosis and 4) synaptic loss (Wisniewski et al., 1997).

The underlying cause of the disease is still in debate. A vast amount of data fortifies the widely accepted amyloid cascade hypothesis. In contrast, the direct link between amyloid deposition and loss of cognitive functions has not been proven convincingly yet. However, an alternative theory which covers the cause and the onset of AD and is experimentally supported has not emerged so far.

Neuropathological features

Atrophy

Post mortem studies show brain volume of AD patients is 10 – 25 % lower than the average volume of control subjects without the disease (de la Monte, 1989; Hubbard and Anderson, 1981). The loss of brain tissue involves both neocortex and cerebral white matter, with the white matter atrophy presumably being a secondary event characterized by a partial loss of myelin sheaths, axons and oligodendroglial cells (Brun and Englund, 1981). Not all brain regions are affected with the same severity. Atrophy in AD is most severe in the temporal lobe, particularly in the medial temporal lobe. The accelerated atrophy of the temporal neocortex, not the hippocampus, in AD patients is associated with a symptomatic onset of dementia (Convit et al., 2000), whereas atrophy of the hippocampus occurs 1 to 2 years before dementia onset (Convit et al., 1997; Fox et al., 1996). The cholinergic subcortical nucleus, nucleus basalis of Meyert is one of the areas that being affected earliest in AD (Toledano and Alvarez, 2004).

Amyloid plaques

The best known histological hallmark of AD is the presence of senile plaques (SP) that are found in the brain of patients suffering from AD. SPs show a topographic distribution that bares some relationship to the stage of the disease. The earliest affected regions are neocortical association areas, especially in temporal and parietal lobes. The amygdala and hippocampus show relatively little amyloid deposition in the early stage of the disease. As the disease progresses, extracellular deposits of amyloid without associated dystrophic neuritis (*i.e.* diffuse plaques) accumulate in the hippocampal dentate gyrus and neostriatum.

Neuritic plaques consist mainly of aggregated amyloid β, but both 1-40 (> 60 %- 70 %) and 1-42 (≤15 %) forms are found, together with minor amounts of other β-amyloid peptides (Aβ1-28, Aβ1-33, Aβ1-34, Aβ3-34, Aβ1-37, Aβ1-38, Aβ1-39) (Golde et al., 2000). Dystrophic neurons are to be found within the amyloid deposits and immediately surrounding them. These neurons are often dilated and are marked with ultrastructural abnormalities such as enlarged lysosomes, numerous mitochondria and paired helical filaments or neurofibrillary tangles. These neuritic plaques are also intimately associated with microglia expressing surface antigens associated with activation and are surrounded by reactive astrocytes displaying abundant microglial filaments (Dickson, 1997). The microglia are usually within and adjacent to the central amyloid core of the neuritic plaque, whereas the astrocytes often surround the outside of the plaque (Selkoe, 1994). Although numerous proteins are associated

with the amyloid deposits in AD, the principal proteinaceous component of the plaque core are aggregates of a <4 kDa polypeptide (Glenner and Wong, 1984), proteolytically derived from a large transmembrane amyloid-β precursor protein (AβPP). AβPP is a single transmembrane protein that is cotranslationally translocated into the endoplasmatic reticulum of the cell via its signal peptide and then post-translationally modified through the secretory pathway. Its half-life is relatively short (~45-60 minutes) (Weidemann et al., 1989). During the trafficking through the secretory pathway, AβPP can undergo a variety of proteolytic cleavages to release secreted derivatives into vesicle lumens and the extracellular space.

The role of AβPP is under extensive research, however, not fully understood yet. The AβPP splice forms of 751 and 770 amino acids are widely expressed in nonneuronal cells throughout the body and also occur in neurons. The 695-residue form is mainly expressed in neuronal cells (Selkoe, 2001). The AβPP of 751 and 770 amino acids contains a 56-residue insert in the middle of the ectodomain encoding a Kunitz-type serine protease inhibitor (KPI). *In vitro* studies confirm that these isoforms can inhibit serine protease such as trypsin and chymotripsin (Sinha et al., 1990). AβPP is able to bind Zn(II) and Cu(II). Zn(II) increases binding of AβPP to heparin and has been shown to potentiate the inhibition of coagulation factor XIa by an AβPP isoform containing a Kunitz-type inhibitory domain (Bush et al., 1993; Smith et al., 1990). AβPP is able to catalyze a reduction of Cu(II) to Cu(I), thus protecting the neuron from potential formation of reactive oxygen species in the presence of Cu(II) (Barnham et al., 2003). A small stretch of amino acids containing the Arg-Glu-Arg-Met-Ser sequence (AβPP328-332) C-terminal to the KPI insertion site has been identified as the active domain responsible for growth promotion and neurite extension in neural cells (Jin et al., 1994; Roch et al., 1994) and for neuronal survival (Yamamoto et al., 1994). It appears that AβPP also is required during an early phase of memory formation (Mileusnic et al., 2000).

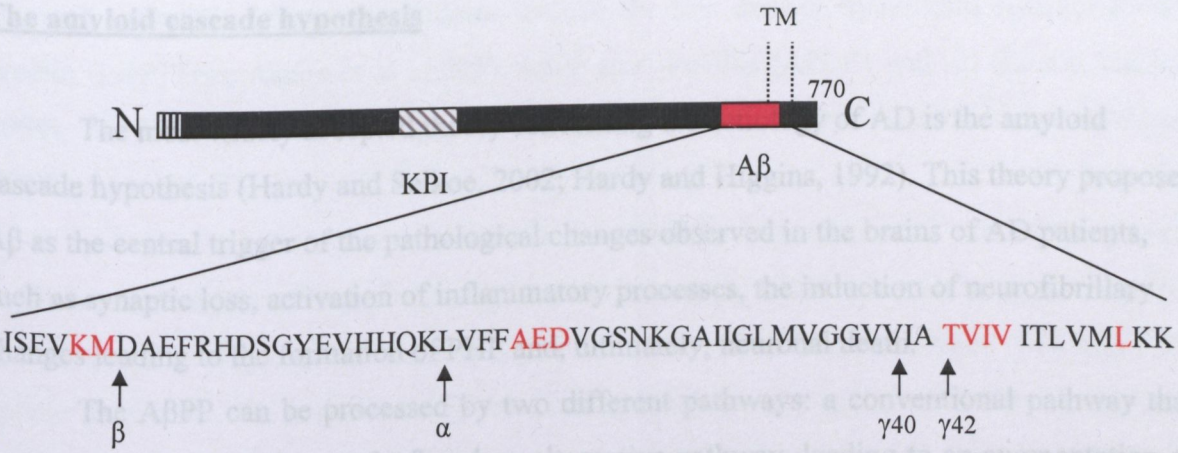

Fig. 1. Schematic diagram of AβPP. Regions of interest (at their correct relative position): the 17-residue signal peptide at the NH_2 terminus (box with vertical lines), two alternatively spliced exons of 56 and 19 amino acids inserted at residue 289 (the first is the KPI domain), the single transmembrane domain (TM) at amino acids 700-723 (vertical dotted black lines). Arrowheads indicate the cleavage sites of α-, β- and γ-secretase. Figure adapted from: (Selkoe, 2001).

Neurofibrillary tangles

Another typical hallmark of AD is the presence of paired helical fragments (PHFs), or neurofibrillary tangles, composed of the microtubule-associated protein tau (τ) in a hyperphosphorilated form, as revealed by immunocytochemical and biochemical analyses. NFTs are mostly found in the nerve cells, accumulating in perikaryon, dendrites and axons (Brion, 1998), but as they are persistent structures following cell death, they are also found extracellularly. The majority of neurons in the typically affected AD brain regions (entorhinal cortex, hippocampus, parahippocampal gyrus, amygdala, frontal, temporal, parietal and occipital association cortices and certain subcortical nuclei projecting to these regions) contain large, non-membrane-bound bundles of abnormal fibres that occupy much of the perinuclear cytoplasm (Selkoe, 2001). Ultrastructurally, these fibres consist of pairs of approximately 10 nm filaments wound into helices with a helical period of ~160 nm as revealed by electron microscopy (Yankner and Mesulam, 1991).

The amyloid cascade hypothesis

The most widely accepted theory concerning the aetiology of AD is the amyloid cascade hypothesis (Hardy and Selkoe, 2002; Hardy and Higgins, 1992). This theory proposes Aβ as the central trigger of the pathological changes observed in the brains of AD patients, such as synaptic loss, activation of inflammatory processes, the induction of neurofibrillary changes leading to the formation of PHF and, ultimately, neuronal death.

The AβPP can be processed by two different pathways: a conventional pathway that does not lead to the release of Aβ and an alternative pathway, leading to an augmentation of the amount of Aβ fragments that are prone to aggregate (Haass and Selkoe, 1993). Three proteases play an important role in the normal and pathological processing of AβPP, *i.e.* α-secretase, β-secretase and γ-secretase. α-secretase is of the family of proteases that process other integral membrane proteins such as transforming growth factor-α and tumor necrosis factor-α (Blacker et al., 2002). It consists of a constitutive component and a regulated component that can be activated via protein kinase C and other second messenger cascades. Several members of the disintegrin and metalloprotease family (ADAM) have been implicated in AβPP-processing: TNF-α converting enzyme (Buxbaum et al., 1998), ADAM10 and ADAM9 (Hotoda et al., 2002) and ADAM17 (Asai et al., 2003). Cleavage by α-secretase is the first step in the non-pathological proteolytic processing of AβPP. The α-secretase cleaves between Lys16 and Leu17 (residues 612 and 613 of AβPP1-695) in the sequence that produces the Aβ itself (Esch et al., 1990), and therefore preludes generation of the Aβ peptide. Processing of AβPP by α-secretase releases a soluble fraction, called αAPPs and leaves an 83 amino acid residue in the cell membrane. The αAPPs might have biological functions in growth regulation and neuroprotection, and in the case of isoforms containing the Kunitz proteinase inhibitor domain, in blood coagulation (Van Nostrand et al., 1992).

β-secretase is a type-1 transmembrane protein containing aspartyl protease activity that cleaves AβPP at the N terminus of the Aβ sequence and is the first prerequisite for generation of Aβ peptides. One enzyme has been identified as the β-secretase, namely BACE-1 (β-site AβPP-cleaving enzyme). Homozygous knockout mice of the BACE-1 gene do not show any generation of Aβ, indicating that this enzyme is indeed the major one with β-secretase activity (Cai et al., 2001). Besides cleavage at the beginning of the Aβ sequence, it also cleaves at Glu11, releasing a shorter form of the Aβ peptide previously identified in plaques (Masters et al., 1985). A close homolog of BACE-1, BACE-2 was identified as well, but this exhibits an α-secretase activity because it cleaves in the middle of the Aβ-domain between phenylalanines 19 and 20 (Fluhrer et al., 2002). The normal physiological substrate of BACE-

1 is not accurately known. Candidates include the low density lipoprotein receptor-related protein (LRP) (von Arnim et al., 2005), AβPP like proteins (APLP1 and -2) (Li and Sudhof, 2004), P-selectin glycoprotein ligand-1 (PSGL-1), and a membrane-bound sialyltransferase (Kitazume et al., 2001).

γ-secretase, which is able to cleave AβPP in the intramembrane part of the protein consists of a complex of four proteins: presenilin, nicastrin, Aph-1 (anterior pharynx-defective phenotype), Pen-2 (presenilin enhancer) (De Strooper, 2003). The presenilins (PS1 and PS2) appear to provide the active core of the protease, but are not sufficient for the proteolytic activity. Mutations in the PS gene can cause the early-onset form of AD by shifting the cleavage site of AβPP two amino acids towards the C-terminus, causing an increase in the Aβ1-42/Aβ1-40 ratio and thereby enhancing oligomer formation of amyloidogenic Aβ1-42. Neuronal cultures derived from PS1 knockout mice showed a severely reduced Aβ generation (De Strooper et al., 1998), and when the PS2 gene was eliminated as well, no Aβ generation was observed at all (Herreman et al., 2000; Zhang et al., 2000). Further proof of the importance of the presenilins in γ-secretase activity came from the observation that all presenilins contain two functionally important and highly conserved aspartate residues within transmembrane domains 6 and 7. As γ-secretase belongs to the aspartyl protease family, the hypothesis that the presenilins and γ-secretase were the same, or at least that the presenilins were a constituent of γ-secretase, was brought up. Indeed, when either of the two aspartates was mutagenized, Aβ generation was abolished (Wolfe et al., 1999). A cofactor identified from the purified complex is nicastrin (Yu et al., 2000). It is a type 1 transmembrane glycoprotein which interacts with both PS1 and PS2, and which has a central role in presenilin-mediated processing of AβPP and some aspects of Notch/glp-1 signaling *in vivo*. When nicastrin was down-regulated in cultured cell lines using small interfering RNA, a massive accumulation of C-terminal fragments of AβPP was seen, together with a marked reduction of Aβ production (Edbauer et al., 2002). Two multipass membrane proteins were isolated during genetic screening for enhancers of a PS-dependent Notch-deficient phenotype in *Caenorhabditis elegans*: Aph-1 and Pen-2 (Francis et al., 2002; Goutte et al., 2002). Besides cleavage of AβPP, γ-secretase is able to process other integral membrane proteins, such as Notch-1 (Moehlmann et al., 2002; Nakajima et al., 2000; Song et al., 1999), which plays a role in gene transcription and cell differentiation, N-cadherin (Marambaud et al., 2003), and ephrinB2 (Georgakopoulos et al., 2006).

Formation and neurotoxicity of Aβ species

The normal physiological pathway of AβPP processing is the secretory pathway, in which AβPP is first cleaved by α-secretase and subsequently by γ-secretase. The first proteolytic cleavage identified is that made by α-secretase and this occurs at 12 amino acids from the amino-terminal of the single transmembrane domain of AβPP (Esch et al., 1990; Sisodia et al., 1990) which results in the release of a large soluble ectodomain fragment called α-APPs. The 83-amino acid COOH-terminal fragment (CTF or p10) may undergo an additional cleavage by γ-secretase, resulting in the formation of p3 and its complementary product p7. The pathological pathway in which AβPP can be processed is the endosomal-lysosomal pathway (Nixon et al., 2000). AβPP is first cleaved by β-secretase to form the N-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Two cleavage products are formed: a secreted soluble ectodomain of AβPP, named βAPPs, and the C99 fragment (Vassar, 2001). C99 is further processed by γ- secretase at several positions between amino acid 39 and 43 of the Aβ sequence. The physical properties and the aggregation behavior of the end-products depend on their length (Burdick et al., 1992).

Initially, investigators proposed that fibrillar Aβ accumulation triggered a pathological cascade that ultimately produced the complete pathological and clinical symptoms of AD. Nowadays it is less clear whether Aβ, deposited as amyloid or some less well characterized β aggregate, initiates the cascade leading to neuronal death and dysfunction. Indeed, small Aβ oligomers, also referred to as Aβ-derived diffusible ligands (Klein, 2002) have been put forward as alternative aggregated forms of Aβ that may mediate toxicity. A recent report, which was received wide publicity, describes Aβ*, a 56 kDa 12-mer Aβ1-42 aggregate, as the ultimate cause for cognitive decline (Lesne et al., 2006). There is also some evidence that intracellular accumulation of Aβ may be neurotoxic (Gouras et al., 2000). To make the picture less clear, recently an alternative aggregation pathway resulting in stable, globular oligomeric species was proposed (Barghorn et al., 2005).

The Aβ-induced pathological cascades leading to neuronal dystrophy and death are not as well defined as the pathways leading to Aβ generation. Multiple pathways are likely to mediate Aβ toxicity. There is evidence that Aβ can be directly neurotoxic, induce oxidative stress, alter calcium homeostasis and incite an inflammatory response in which all typical features of an inflammatory response to an insult are present: There is activation of the complement system, the concentration of chemokines and cytokines such as IL-1β, IL-6, TNF-α, TGF-β and macrophage inflammatory protein-1α (MIP-1α) seem to be upregulated, the level of cyclooxygenase-2 (COX2) is increased in AD brain and there is an increased β2-

integrin LFA-1 expression by microglia cells, as well as other acute phase proteins (Akiyama et al., 2000). These events might be mediated by direct interaction of Aβ aggregates with cellular membranes or by the binding of Aβ to microglial and neuronal cellular receptors. Indeed, Aβ1-42 is able to bind to several neuronal membrane proteins (insulin receptor, serpin complex receptor, α7 nicotinic acetylcholine receptor, integrin β1, receptor for advanced glycosylation end products, collagen-like Alzheimer amyloid plaque component precursor, N-methyl-D-aspartate receptor, AβPP, P75 neurotrophin receptor) (for review, see (Verdier et al., 2004). Once internalized, Aβ has been shown to bind to numerous vital housekeeping enzymes (Verdier et al., 2005).

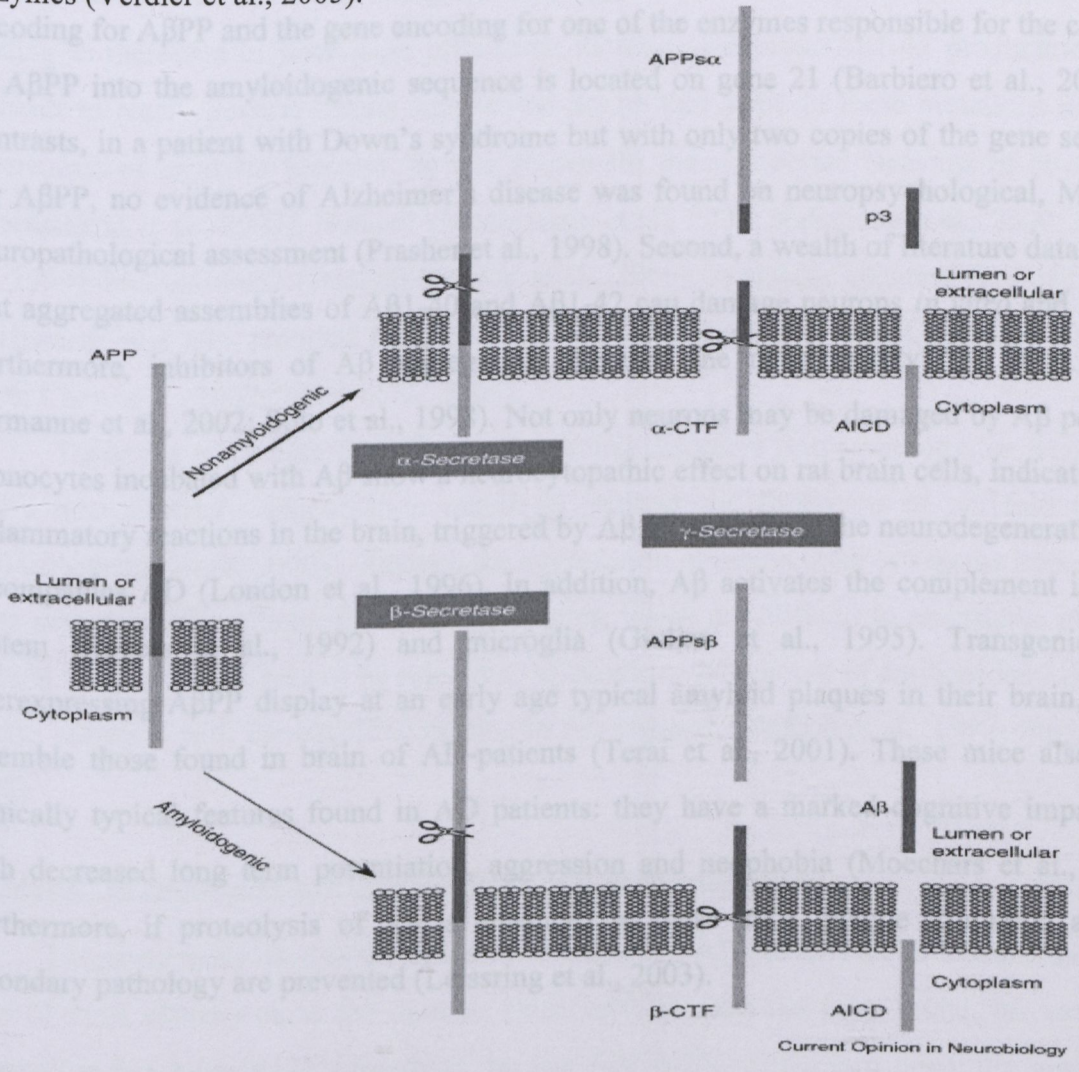

Fig. 2.: Proteolytic processing of AβPP by the secretases. The majority of APP is processed in the nonamyloidogenic pathway (thick arrow); APP is first cleaved by α-secretase within the Aβ domain (darker shaded region), leading to APPsα secretion and precluding Aβ generation. α-carboxy terminal fragment (CTF) is then cleaved by γ-secretase within the membrane, releasing the p3 peptide and AICD. Alternatively, amyloidogenesis (thin arrow) takes place when AβPP is first cleaved by β-secretase, producing APPsβ. Aβ and AICD are generated upon cleavage by γ-secretase of the β-CTF fragment retained in the membrane. Scissors indicate the cleavage sites of α-, β- and γ–secretase. Figure from (Wilquet and De Strooper, 2004).

Data supporting the amyloid cascade hypothesis

Several pieces of evidence support the amyloid cascade hypothesis: first, people with mutations in the AβPP, PS1 and PS2 genes are associated with elevated levels of Aβ proteins (Scheuner et al., 1996), suffering from the inherited form of AD, suggesting that Aβ plays an important role in the onset of the disease. Moreover, patients with trisomy 21 (Down's syndrome) virtually all develop senile plaques and by the age of 40 have sufficient neuropathology for a diagnosis of AD (Head and Lott, 2004). These patients all have 3 alleles of chromosome 21 in each cell, which leads to a higher level of Aβ proteins, as the gene encoding for AβPP and the gene encoding for one of the enzymes responsible for the cleavage of AβPP into the amyloidogenic sequence is located on gene 21 (Barbiero et al., 2003). In contrasts, in a patient with Down's syndrome but with only two copies of the gene sequence for AβPP, no evidence of Alzheimer's disease was found on neuropsychological, MRI and neuropathological assessment (Prasher et al., 1998). Second, a wealth of literature data reports that aggregated assemblies of Aβ1-40 and Aβ1-42 can damage neurons *in vitro* and *in vivo*. Furthermore, inhibitors of Aβ aggregation attenuate the neurotoxicity (Du et al., 2003; Permanne et al., 2002; Soto et al., 1998). Not only neurons may be damaged by Aβ peptides. Monocytes incubated with Aβ show a neurocytopathic effect on rat brain cells, indicating that inflammatory reactions in the brain, triggered by Aβ, play a role in the neurodegeneration that accompanies AD (London et al., 1996). In addition, Aβ activates the complement immune system (Rogers et al., 1992) and microglia (Giulian et al., 1995). Transgenic mice overexpressing AβPP display at an early age typical amyloid plaques in their brain, which resemble those found in brain of AD-patients (Terai et al., 2001). These mice also show clinically typical features found in AD patients: they have a marked cognitive impairment with decreased long term potentiation, aggression and neophobia (Moechars et al., 1999). Furthermore, if proteolysis of Aβ is enhanced in these mice, plaque formation and the secondary pathology are prevented (Leissring et al., 2003).

Data against the amyloid cascade hypothesis

Transgenic animals are commonly used for studying AD. The most widely used Tg2576 line overexpresses human AβPP containing the double Swedish mutation K670NM671L. Tg2576 mice develop cerebral Aβ deposits and spatial memory deficits in an age dependent manner, however no neurofibrillary tangle formation or significant neuronal loss in CA1 (Hsiao et al., 1996; Irizarry et al., 1997). Recently, a triple Tg model published by Oddo and coworkers based on comparable overexpression of mutant human APP, PS1 and tau

has been published, which develops early deficits in long-term synaptic plasticity that correlate with accumulation of intraneuronal Aβ followed by amyloid plaques and NFT formation in an age-dependent fashion (Oddo et al., 2003). Interestingly, extracellular Aβ deposition precedes tau pathology by several months in this latter Tg line, which seems to favor the amyloid cascade pathogenic hypothesis. However, no human yet has been found carrying these triple mutations.

The exact molecular mechanism by which FAD-linked PS1 pathogenic mutations affect AβPP processing in the diseased human brain, or even in cell culture or animal models is still in debate. A consensus about the near one dozen FAD linked point mutation in PS1 leading to increased in γ-secretase efficiency; therefore increased Aβ1-42 production seems difficult to reach. Several results support the notion that some of these mutations lead to a severe loss of catalytic function, as was shown for the γ-secretase cleavage of N-cadherin (Marambaud et al., 2003), Notch-1 (Moehlmann et al., 2002; Nakajima et al., 2000; Song et al., 1999), and ephrinB2 (Georgakopoulos et al., 2006). A similar apparent loss-of-function mechanism was also reported for AβPP processing at the ε-cleavage site (Bentahir et al., 2006; Chen et al., 2002; Wiley et al., 2005).

There are more than 20 different dementing disorders (tau pathology, or tauopathy), where neurofibrillary degeneration manifests without the formation of amyloid plaques (Delacourte and Buee, 2000). Although the aggregation of intracellular tau seems important in these dementias, the role of diffusible Aβ species, which are difficult to investigate in a post mortem brain material, cannot be ruled out. In addition, the amount of amyloid load does not always correlate with the cognitive decline in AD patients (Mufson et al., 1999; Nagy et al., 1995).

Treatment of Alzheimer's disease: current and future therapies

Drugs currently used in the therapeutic scheme of AD patients are all symptomatic and none of them actually cures the disease. Until now, no cure has been found, but extensive progression has been made throughout the last five years, which will hopefully lead to an effective cure.

Donepezil (Aricept®), galantamine (Reminyl®) and rivastigmine (Exelon®) are all cholinesterase inhibitors which lead to an increase of acetylcholine in the brain and therefore can aid the patient (Cutler and Sramek, 2001). These products stabilize the memory decline for a variable period (typically 6 to 12 months). These drugs are effective in delaying the deteriorating symptoms in patients with mild to moderate AD, but are not effective any more in the end. The clinical usefulness of these drugs is doubtful, but to date these are the only

products that can influence the progress of the disease in an early stage. Since AD is often accompanied by symptoms of depression and anxiety, anti-depressants and anxiolytics are frequently added to the therapeutic scheme of the patient.

Psychotropic medications play a critical role in the management of behavioral disturbances of patients with AD. Relatively few psychotropic compounds have been tested specifically in AD populations. Recent double-blind, placebo-controlled trials have established the efficacy of the atypical antipsychotics risperidone and olanzapine for the treatment of psychosis and agitation in patients with AD (Street et al., 2001). Vitamin E and selegiline have been shown to reduce the rate of decline of functions in patients with AD. Evidence to support the use of other antioxidants, anti-inflammatory agents, or herbal medications such as *Ginkgo biloba* is insufficient to recommend use as standard therapies (Luo, 2001). Estrogen in high doses has been shown to improve cognition in postmenopausal women with AD (Asthana et al., 2001).

Blocking N-methyl-D-aspartate (NMDA) receptor is an other approach in the current treatment of AD. Memantine is a new symptomatic drug that recently has been approved by the FDA. It is a noncompetitive NMDA receptor antagonist which, after binding to the postsynaptic NMDA-receptors, can prevent glutamate excitotoxicity which leads to neuronal calcium overload and ultimately to cell death (Wilkinson, 2001), but does not prevent physiological activation of the receptor.

On the other hand, Weggen et al. reported that administration of certain non-steroidal anti-inflammatory drugs (NSAID) in the appropriate doses, leads to a reduction in Aβ1-42 formation independently of their influence on cyclooxygenase activity (Weggen et al., 2001). Flurbiprofen is a NSAID consisting of two enantiomers able to lower Aβ1-42 *in vivo* by targeting γ-secretase (Eriksen et al., 2003). By using only the R enantiomer of the drug, R-flurbiprofen retains the amyloid lowering ability without the accompanying COX inhibition and related ulcer-causing effects. Doses used to obtain this effect are very high and further research is necessary to obtain more accurate data on this matter.

All these therapies remain symptomatic and do not eliminate the cause of the disease, namely the wrong splicing of AβPP and subsequent deposition of Aβ in plaques. New therapies that interfere with this process are momentarily being investigated. One example is the use of induction of Aβ immunity (Frenkel et al., 2000; Schenk et al., 1999; Schenk et al., 2000). Injecting transgenic AD mice with the purified Aβ protein leads to the development of antibodies against amyloid protein. Studies showed that this immunization therapy not only inhibited the generation of new plaques, but could even lead to the removal of already existing plaques (Schenk et al., 1999). DeMattos and coworkers (DeMattos et al., 2002)

showed that the peripheral administration of a monoclonal antibody (m266) which is directed against the central domain (residues 13-28) of Aβ1-42, can reduce the cerebral Aβ burden in mice. Active immunization with synthetic intact Aβ1-42 or conjugated fragments of Aβ42 has also been evaluated in humans while techniques for passive immunization with human anti-Aβ monoclonal antibodies (mAβ) are approaching clinical development. Phase II clinical trials of the active immunization study had to be halted because some patients developed fatal aseptic meningo-encephalitis (Orgogozo et al., 2003).

Blocking of β- and/or γ-secretase is an option, which is being pursued by several groups, however the biological function of BACE-1 has not yet been determined and the question arises which unwanted consequences blocking BACE-1 will have. However, in BACE-1 knock-out mice, there is no generation of any Aβ and these mice appear to be healthy without perceivable neurological or behavioral abnormalities (Luo et al., 2001; Roberds et al., 2001). In contrast, blockade of gamma secretase caution is needed because of its function in Notch processing which is of great importance for cell survival. Notch signaling pathway does play very important role in cell fate decisions during embryogenesis, and in adult life, as it regulates haematopoiesis (Suzuki and Chiba, 2005), neurite outgrowth and maintenance (Berezovska et al., 1999). Full blockade of γ-secretase will certainly interfere with this pathway.

Since the underlying cause of the disease seems to be the aggregation of the misfolded Aβ protein species, approaches targeting the folding process and aggregation may be beneficial. Aβ peptides undergo a conformational change from the soluble α-helical/random coiled structure to a self aggregation-proned β-hairpin structure. Stabilizing the α-helical conformation, or destabilizing the β-sheet structure is of great promise. Indeed, there are several compounds, called β-sheet breakers or amyloid aggregation inhibitors (BSB, AAI (Talaga, 2001)). Two main types of BSB's are known currently: non-peptide and peptide-based compounds. Non-peptide BSB's are generally planar molecules with a hydrophobic bi- or tricyclic scaffold. Among them, there are natural biomolecules, like melatonin (Pappolla et al., 1998), nicotine (Salomon et al., 1996), curcumin (Ono et al., 2004; Yang et al., 2005), or artificial compounds, like 4'deoxy-4'iodorubicin derivates (IDX, (George and Howlett, 1999), benzofuran derivates, (Howlett et al., 1999), and the antibiotic rifampicin (Tomiyama et al., 1994). The native sequence of Aβ16-21, KLVFFA has been reported to be a key element of the aggregation process (Hilbich et al., 1992; Tjernberg et al., 1999), therefore peptide-based BSB's are based mainly on this part of Aβ molecules. Tjernberg et al. published the AAI effect of KLVFF fragment (Tjernberg et al., 1996). Soto et al. modified the LVFFA sequence to LPFFD, which proved to interfere with the aggregation of Aβ1-42 (Soto et al., 1996; Soto

et al., 1998). Studies have shown that fragment Aβ31-35 also plays an important role in the toxicity of Aβ1-42 (Yan et al., 1999). An analogue of Aβ30-34 was able to protect against the intracellular Ca^{2+} level increasing effect of Aβ1-42 *in vitro* (Laskay et al., 1997). However, these compounds have to be selective to the toxic forms of amyloid assemblies, since non-toxic aggregates have been reported in mammals recently (Fowler et al., 2006).

A short overview of glutamate receptors

The excitatory amino acids glutamate (Glu) and aspartate are the most abundant of all amino acids in the brain, and are known to elicit fast excitatory responses in neurons in various species. The neurotransmitter nature of these amino acids is now widely recognized, being the principal neurotransmitter for fast excitatory signaling in brain. Glutamate is a non-essential amino acid that does not cross the blood-brain barrier. Glu is synthesized directly in the brain from α-ketoglutarate in the mitochondrial compartment of Glu-erg nerve terminals either through transamination of aspartate or by conversion from glutamine (for review see (Tapiero et al., 2002). Glutaminergic neurotransmission is terminated by Glu uptake into neurons or glial cells through specific Glu transporters, namely excitatory amino acid carriers (EEAT's (Shigeri et al., 2004). The receptors of Glu are regarded to be almost ubiquitous (Orrego and Villanueva, 1993), and may be divided into two large families: metabotropic (mGluR) and ionotropic Glu receptors. The mGluR's are G-protein coupled and eight receptor subtypes have been cloned so far (mGluR1-8, (Pin and Acher, 2002).

Ionotropic EAA receptors are multi-subunit transmembrane proteins that consist of a conducting pore embedded in the cell membrane and various binding sites on the extracellular surface of the receptor. The ionotropic receptors are named after the specific agonist to which they respond. The α-amino-3-hydroxy-5-methyl-isoxazole proprionic acid (AMPA) receptor, formerly known as the quisqualate receptor, is associated with a cation channel that is nonselective with respect to Na^+ and K^+ ions. Furthermore, it has a certain degree of Ca^{2+} permeability which is determined by the GluR2 subunit (Jonas et al., 1994). AMPA receptor mediated currents exhibit fast kinetics; with rapid onset, offset and desensitization. The AMPA receptors are widely distributed in the brain, with high density in the hippocampus and olfactory tubercle (Petralia and Wenthold, 1992). This receptor subtype is considered to be major mechanism for fast excitatory signaling in the brain (Wisden and Seeburg, 1993). Currently there are four different subunits described for AMPA receptors termed GluR1 through GluR4, each of which occurs in two variants, "flip" and "flop" which are the result of alternative gene splicing. Kainate receptors are similar in ion gating and kinetics to AMPA

receptors and may be formed from five subunits designated GluR5, GluR6, GluR7, KA1 and KA2. (Petralia et al., 1994a).

The N-methyl-D-aspartate (NMDA) receptors exhibit comparatively slower kinetics than AMPA and kainate receptors and also show significant permeability to Ca^{2+} ions in addition to Na^{+} and K^{+}. NMDA receptors may be composed of various subunits, e.g. NR1 and NR2A-NR2D. Activation of NMDA receptors by glutamate has been shown to require concomitant binding of glycine to a specific glycine binding site. In addition, NMDA receptors are blocked by relatively low concentrations of Mg^{2+} via interaction with a binding site within the ion channel complex, and a certain level of depolarization is needed for the Mg^{2+} block to be removed. The NMDA receptors are widely distributed in brain, with high densities found in cortical regions and hippocampus (Petralia et al., 1994b). All of these receptors play a key part in the processes of synaptic plasticity, like long term potentiation and long term depression.

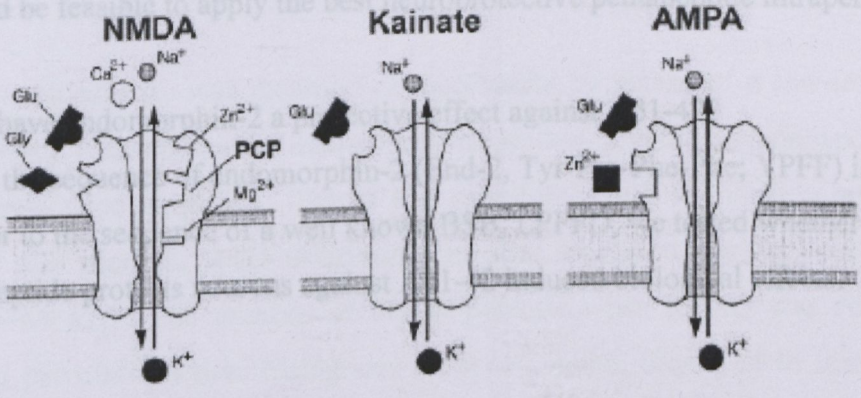

Fig. 3.: Schematic drawing of the three major types of ionotropic glutamate receptors. Figure adapted from Kandel et al., 1991 .

Aims

The main goal of this Ph.D. work was to elucidate the effect of Aβ1-42 on synaptic transmission, which may underlie the rapid synaptotoxic effect of the peptide. Employing short peptides, we also sought for strategies, by which neuroprotection could be achieved. Therefore, we aimed to answer the following questions:

- What effect does Aβ1-42 exert on rapid synaptic function, more specifically on postsynaptic ionotropic glutamate receptors?

- Which sequence of Aβ1-42 might interfere with Aβ1-42 induced neuromodulation?

- Is there a difference between the mechanisms of protection of pentapeptides?

- Would be feasible to apply the best neuroprotective pentapeptide intraperitoneally?

- Does have endomorphin-2 a protective effect against Aβ1-42?
  Since the sequence of endomorphin-2 (End-2, Tyr-Pro-Phe-Phe; YPFF) is highly similar to the sequence of a well known BSB, LPFFD, we tested whether the tetrapeptide protects neurons against Aβ1-42 induced biological effects.

Materials and Methods

In vivo extracellular single-unit electrophysiology

Extracellular single-unit recordings were made in chloral hydrate-anesthetized male Wistar rats weighing 300-360g. After the head of the animal had been mounted in a stereotaxic frame, the skull was opened above the hippocampus (antero-posterior: -2.8 to -3.8 from bregma; lateral: 2 mm on either side from the midline), and the dura mater was carefully removed. Structures were localized according to the stereotaxic atlas of Paxinos and Watson (1986). All efforts were made to minimize animal suffering. The principles of laboratory animal care (NIH publication No. 85-23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed.

Extracellular recordings

Single-unit activity was recorded extracellularly by means of a low-impedance (< 1 MΩ) 7 μm carbon fiber-containing microelectrode (Kation Scientific, Minneapolis, MN). The action potentials were amplified, filtered and monitored with an oscilloscope. A window discriminator was used for spike discrimination. The amplified signals were sampled and digitalized at 50 kHz. The number of action potentials per second was counted by the computer and peristimulus time histograms were calculated, displayed in line and digitally stored for off-line analysis. Iontophoretic drug delivery and experimental data collection were performed by a multifunction instrument control and data acquisition board (National Instruments PCI-1200) situated in a computer.

Solutions

The drug barrels of the combined recording/iontophoresis electrode contained one or other of the following solutions: 100 mM NMDA Na salt (pH 8.0), 10 mM AMPA hydrobromide (pH 8.0), 20 mM KA in 180 mM NaCl (pH 8.0), 2.5×10^{-4} M DAMGO/End-2 dissolved in saline (pH 6.4), 2.5×10^{-4} M pentapeptide (RIIGL, LPYFDa, RVVIA, FRHDS) dissolved in pH 6.4 saline, a mixture containing Aβ1-42 (5×10^{-5} M) and DAMGO/End-2 (2.5×10^{-4} M), a mixture containing Aβ1-42 (5×10^{-5} M) and one of the above pentapeptides (pH 6.4), 5×10^{-5} M Aβ1-42 (pH 6.4). The mixture solutions were stored for 24 h at 4 °C. To lessen the degree of aggregation, Aβ1-42 and the mixture solutions were sonicated (Merck Eurolab

120 W apparatus) for 15 min prior to use. All substances were purchased from Sigma Aldrich, (Budapest, Hungary), with the exception of Aβ1-42, End-2 and the pentapeptides, which were synthetized in-house by a solid-phase procedure involving the use of Wang resin and Fmoc chemistry.

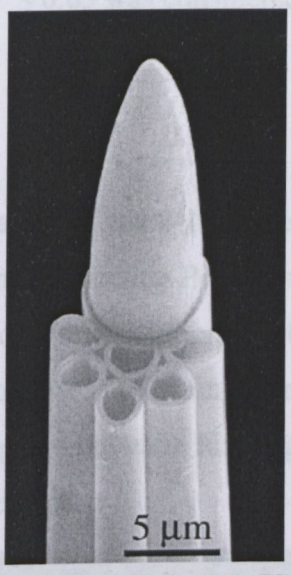

Fig.. 4.: Scanning electromicrograph of the Carbostar 7S multibarrel electrode tip. Courtesy of Kation Scientific.

Iontophoresis

Excitatory agents were ejected with negative iontophoretic currents ranging from 2 to 100 nA. Retaining currents in the interval 2–21 nA of opposite direction were used. The pentapeptides, End-2 and DAMGO were ejected at +100 nA for 3 minutes, Aβ1-42 and the mixtures for 1 min at –0.5 µA. Cells were excited by the brief (5s) repetitive ejection of NMDA and AMPA or NMDA and KA. The ejection current was selected so that the maximum firing rate fell between 30 and 80 spikes/s. The interval between two excitation epochs was 120 s. When NMDA alone was used for excitation, ejection occurred every minute. The peristimulus time histograms of the neurons were recorded. After establishment of a stable control (at least four successive peaks), the solution to be examined was ejected.

Data analysis

Statistical evaluations were performed by using the total number of spikes evoked during each excitation epoch by iontophoretic application of an excitatory agent. The background neuronal discharge was calculated by averaging a 15s period of ongoing activity preceding each excitation epoch, and this value was subtracted from all evoked responses.

The total spike number during each excitation epoch was calculated and expressed as a percentage of the mean (± SEM), and compared statistically with the data obtained after peptide application by using one-way analysis of variance (ANOVA, with the Bonferroni test for *post hoc* analysis). A P value of ≤ 0.05 was considered significant.

Histology

In order to confirm the location of the cells under study, the last recording site was marked at the end of the experiment by ejecting Pontamine Sky Blue with 5 μA for 20 min. The brains were removed and fixed with a solution of 10% paraformaldehyde and glucose. Forty μm thick serial coronal sections were cut using a freezing-microtome and stained with neutral red. The marked recording positions were histologically determined in frontal frozen sections by means of stereotaxic coordinates

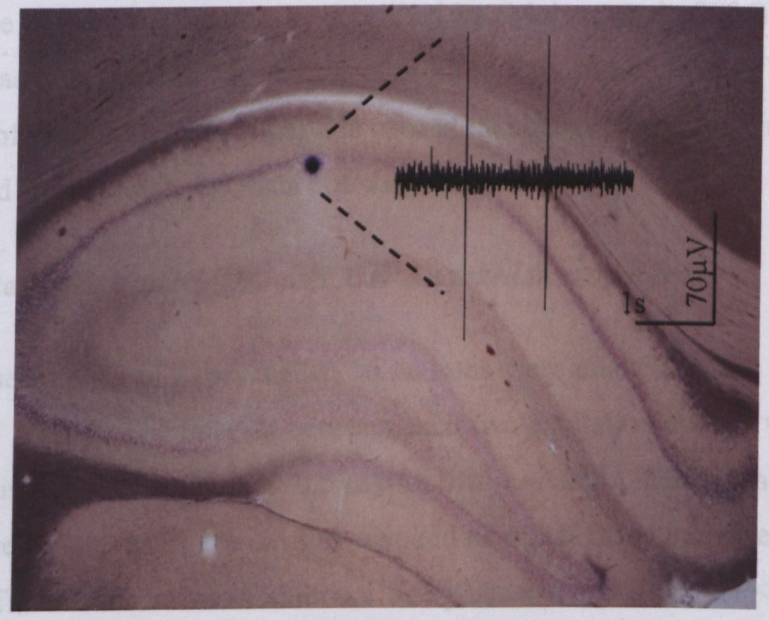

Fig. 5.: Histological verification of the recording site. Forty-micrometer thick slices were prepared with a microtome and then stained with neutral red. Pontamine Sky Blue marks the position of the electrode tip in the CA1 hippocampal region.
Inset: representative single-unit action potentials recorded from the marked site.

Results

Effects of Aβ1-42 on the ionotropic Glu receptor mediated neuronal firing

Experiments were carried out on a total of 61 hippocampal CA1 neurons recorded from 45 anesthetized rats. All these cells, which had little or no spontaneous activity, responded to NMDA, AMPA or KA applied iontophoretically prior to the application of Aβ1-42.

Effect of Aβ1–42 on the NMDA-evoked response

We have examined the effect of Aβ1-42 ejection on the NMDA-evoked responses on one neuron, without utilizing other excitatory agent. NMDA was ejected at every minute. The percent change in the number of NMDA-evoked spikes after Aβ1–42 was 267% ±19% (n = 14) as compared with the pre-Aβ1–42 control. The enhancement of the NMDA triggered response peaked between 8 and 27 min and did not return to the control level within the 45-min time-frame of the experiments, as described by (Molnar et al., 2004), although the level of responses had decreased to 155 ± 28% after 18 to 24 min in 5 cells of the 14. In contrast, iontophoresis of the saline without peptide caused no change in the baseline activity or in the NMDA-evoked firing rate (n = 6, data not shown).

Aβ1-42-mediated effects on NMDA- and AMPA-evoked neuronal firing

In the second set of experiments, 16 neurons were excited by the alternating ejection of NMDA and AMPA. The interval between each two excitation epochs was 120 s. Aβ1-42-containing solutions were ejected at -0.5 μA for 1 min. After a stable control period, Aβ1-42 was administered. As a result, the NMDA- evoked neuronal firing increased up to a maximum of 260±28%. The enhancement of neuronal firing became significant ($P<0.05$) following the second or third NMDA excitation (5-7 min after Aβ1-42 ejection), and the firing frequency remained at a high level throughout the timeframe of the experiment. In contrast, the AMPA responses decreased significantly after Aβ1-42 application in all of the measured cells. Two types of cells were found on the base of temporal change of firing frequency. In 5 cells, the AMPA-mediated responses started to decrease only 23-25 minutes after Aβ1-42 application, not following the temporal changes in the NMDA-evoked firing (n=5). Other cells responded to Aβ1-42 with a sharp, immediate decrease in AMPA evoked firing (n=6), accompanied by the previously described trend of an increase in the NMDA-elicited responses. In both sets of cells, the AMPA responses almost completely disappeared in 35-37 min (9.5±5.5%; $P<0.05$). Iontophoresis of the vehicle alone (saline, pH 6.4) caused no change in the NMDA and AMPA-mediated responses (n=5; data not shown).

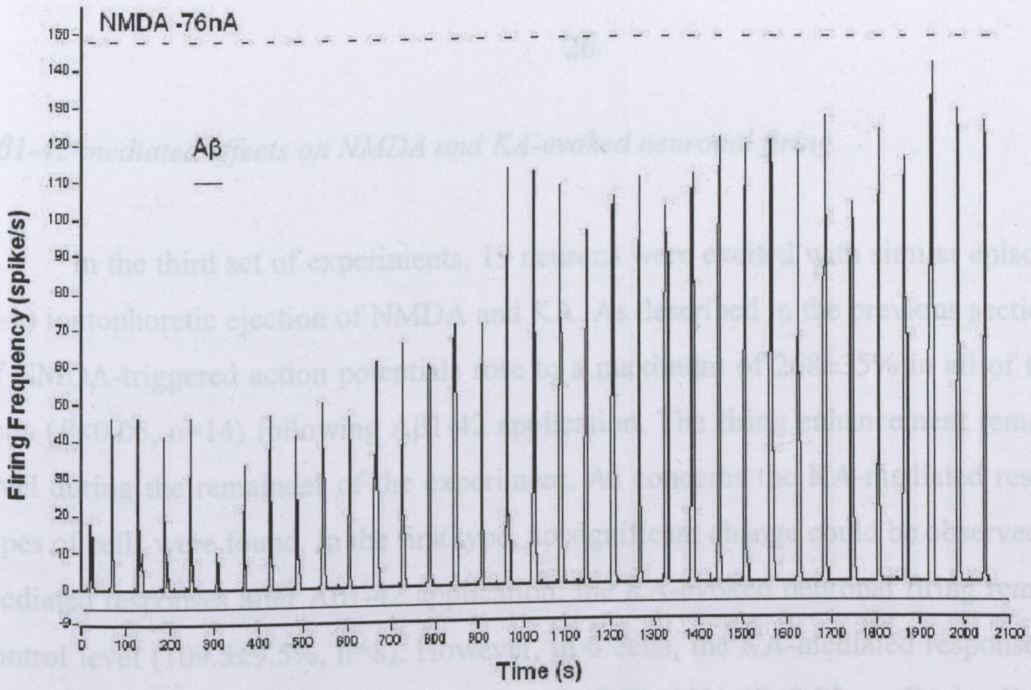

Fig. 6.: Effect of Aβ1–42 on the NMDA-evoked responses of a CA1 rat hippocampal neuron in vivo. Aβ1–42 (50 μM capillary concentration) application is denoted. The total number of spikes evoked during each epoch of excitation per minute before and after peptide application (Aβ1–42) was compared. Aβ1–42 was ejected at -0.5 μA for 1 min.

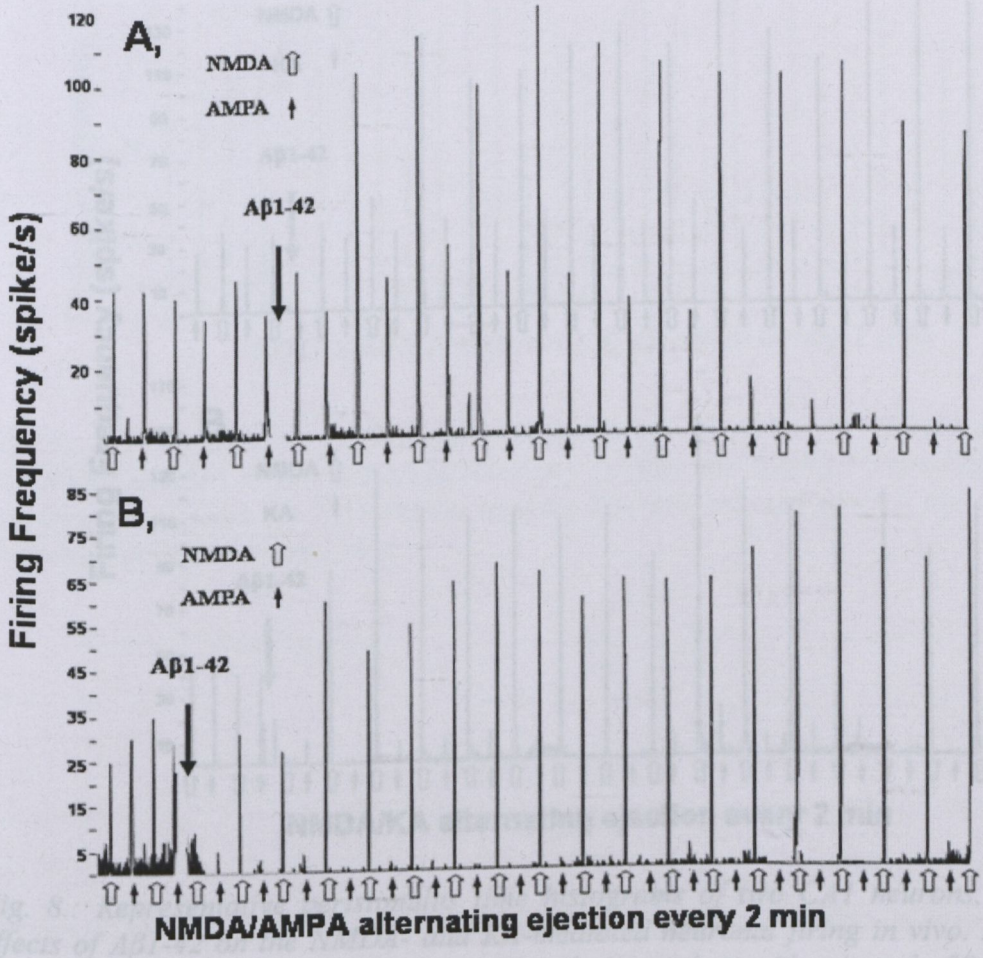

Fig. 7.: Representative peristimulus histograms showing the effects of Aβ1-42 on the NMDA- and AMPA-evoked firing on CA1 neurons in vivo. NMDA and AMPA were ejected at -46 nA and -87 nA (Panel A), and at -24 nA and -39 nA (Panel B), respectively. Aβ1-42 was applied at -0.5 μA for 1 min. The interval between two excitation epochs was 120 s. Note the decrease in spontaneous activity of the cells after peptide application.

Aβ1-42-mediated effects on NMDA and KA-evoked neuronal firing

In the third set of experiments, 19 neurons were excited with similar episodes of brief (5-s) iontophoretic ejection of NMDA and KA. As described in the previous section, the level of NMDA-triggered action potentials rose to a maximum of 268±35% in all of the recorded cells ($P<0.05$, n=14) following Aβ1-42 application. The firing enhancement remained at this level during the remainder of the experiment. As concerns the KA-mediated responses, two types of cells were found. In the first type, no significant change could be observed in the KA-mediated responses after Aβ1-42 application: the KA-evoked neuronal firing remained at the control level (109.5±9.5%, n=8). However, in 6 cells, the KA-mediated responses decreased immediately after Aβ1-42 ejection to a level of 28±12% ($P<0.05$, n=6). Application of the vehicle without Aβ caused no change in the NMDA and KA-mediated responses (n=5; data not shown).

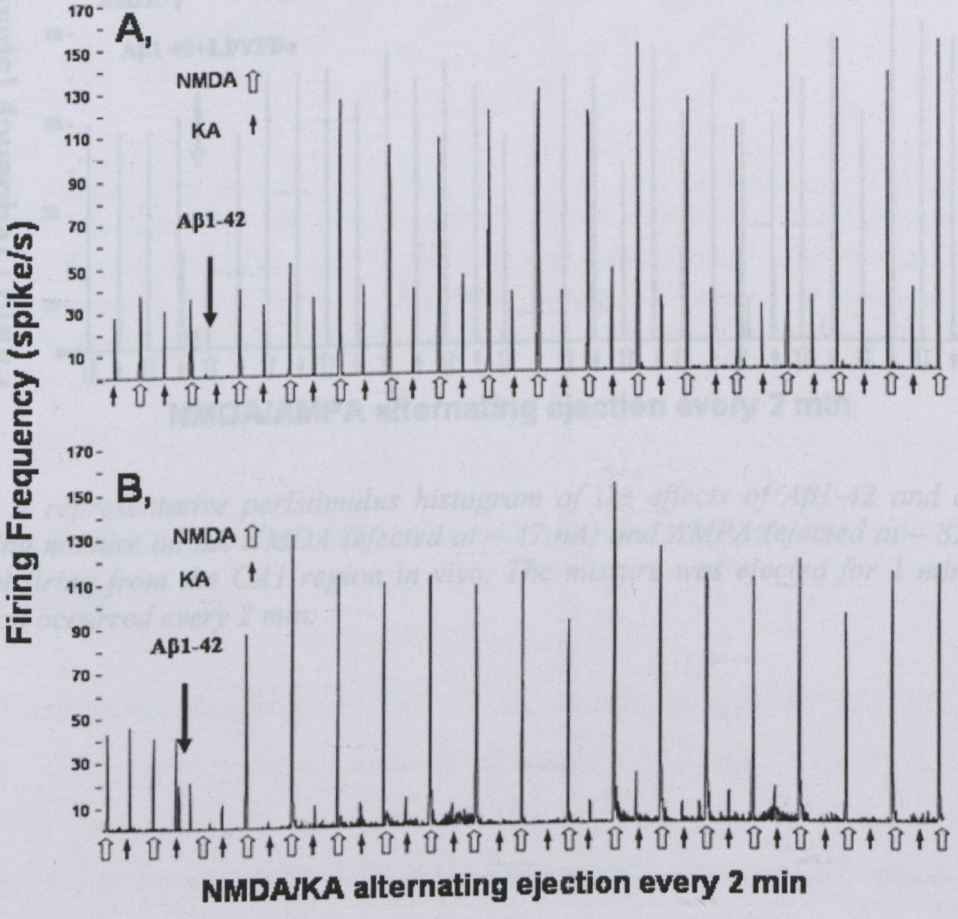

Fig. 8.: Representative peristimulus time histograms of two CA1 neurons, displaying the effects of Aβ1-42 on the NMDA- and KA-mediated neuronal firing in vivo. NMDA and KA were ejected at -84 nA and -63 nA (Panel A), and at -41 nA and -59 nA (Panel B), respectively. Aβ1-42 was applied at -0.5 μA for 1 min. Excitation occurred every 2 min. Aβ1-42 caused no change in the KA responses in the first group of cells (n=8, Panel A), while KA responses disappeared almost completely in the second group (n=6, Panel B).

Effects of LPYFDa and Aβ1-42 mixture on NMDA and AMPA-evoked neuronal firing

To verify the Aβ1-42 specificity in the attenuation of AMPA responses, we employed a protective pentapeptide, LPYFDa. It has been reported that this pentapeptide protects neurons against the fEPSP attenuating and cell-viability decreasing action of Aβ1-42 *in vitro (Szegedi et al., 2005a).* A mixture of LPYFDa ($2.5x10^{-4}$ M) and Aβ1-42 ($5x10^{-5}$ M) was applied in a 5:1 molar ratio at -0.5 μA for 1 min. Ejection of this mixture did not significantly change the level of either the NMDA or the AMPA-evoked responses (n=6). The maximum in the NMDA-mediated responses was 115±12%, while that in the AMPA-elicited firing was 125±18%.

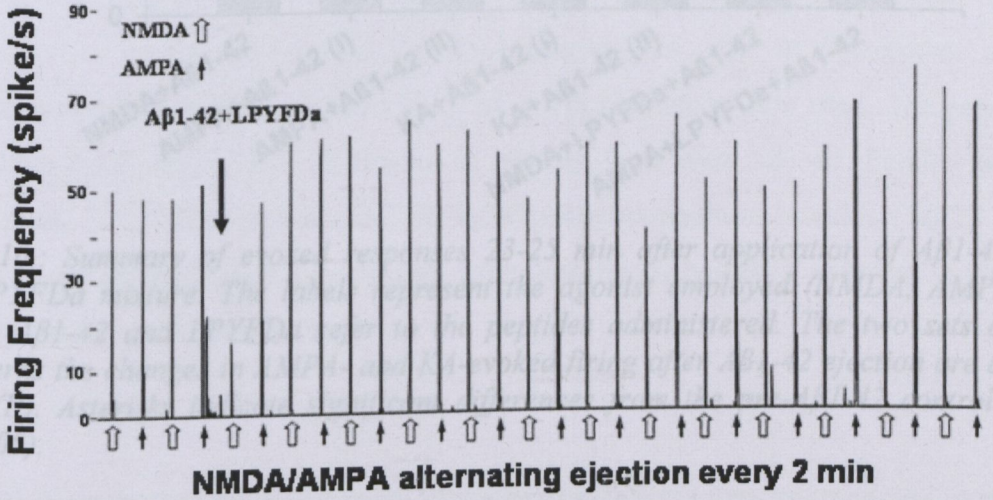

Fig. 9.: A representative peristimulus histogram of the effects of Aβ1-42 and an LPYFDa-containing mixture on the NMDA (ejected at – 47 nA) and AMPA (ejected at – 82 nA)-evoked neuronal firing from the CA1 region in vivo. The mixture was ejected for 1 min at -0.5 μA. Excitation occurred every 2 min.

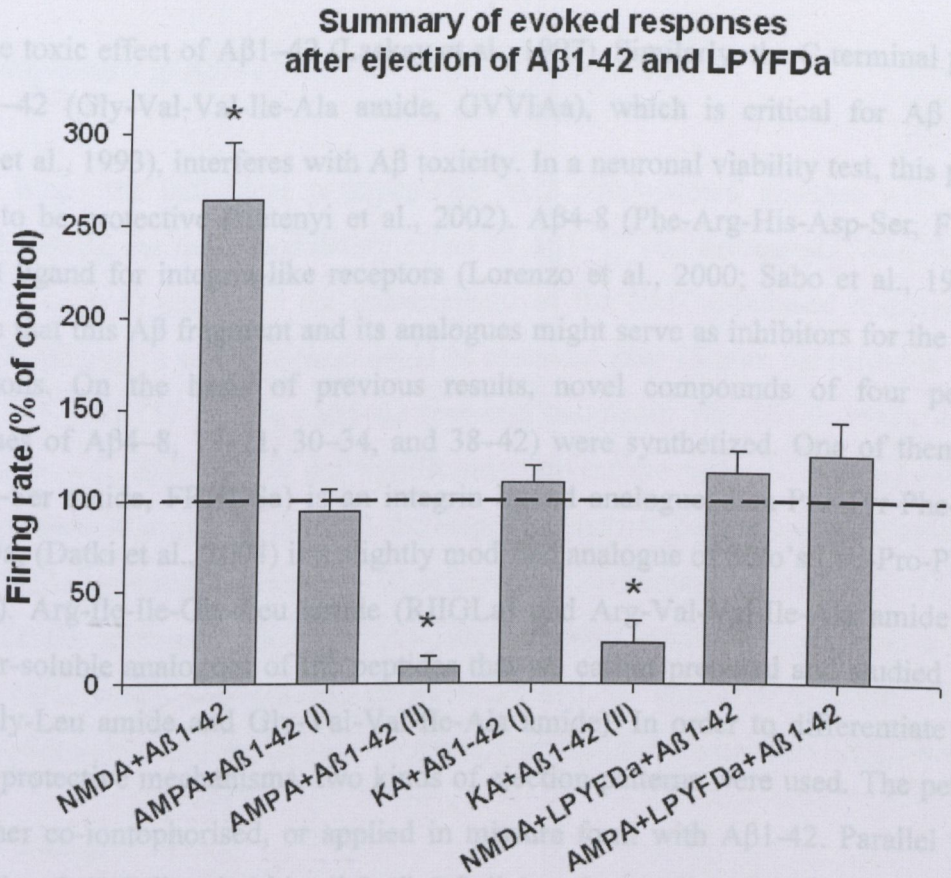

Fig. 10.: Summary of evoked responses 23-25 min after application of Aβ1-42 or Aβ1-42/LPYFDa mixture. The labels represent the agonist employed (NMDA, AMPA or KA), while Aβ1-42 and LPYFDa refer to the peptides administered. The two sets of cells as concerns the changes in AMPA- and KA-evoked firing after Aβ1-42 ejection are denoted (I) and (II). Asterisks indicate significant differences from the pre-Aβ1-42 control (ANOVA, $P<0.05$).

Aβ1-42 derived pentapeptides protect against Aβ1-42 induced enhancement of NMDA triggered firing

Short peptide fragments of Aβ1-42 are thought to interfere with the aggregation properties of Aβ peptides. Since the biological activity of Aβ1-42 is strongly dependent of its aggregation state, inhibiting aggregation would be a feasible way in the combat against Alzheimer's disease. Our aim was to find pentapeptides, which protect against the NMDA response enhancing effect of Aβ1-42 *in vivo*, and to differentiate between the possible inhibitory mechanisms. For the identification of peptide recognition sites, our group has designed different pentapeptides spanning the entire Aβ1–42 sequence. Peptide design was supported by theoretical considerations and our former results. Our research group found that Aβ31–35 (Ile- Ile-Gly-Leu-Met; IIGLM) is neurotoxic (Penke and Baranyi, 1994), however, the tetrapeptide propionyl-Ile-Ile-Gly-Leu amide (Pr- IIGLa) protects glial and neuronal cells

from the toxic effect of Aβ1–42 (Laskay et al., 1997). Similarly, the C-terminal pentapeptide of Aβ1–42 (Gly-Val-Val-Ile-Ala amide, GVVIAa), which is critical for Aβ aggregation (Jarrett et al., 1993), interferes with Aβ toxicity. In a neuronal viability test, this pentapeptide proved to be protective (Hetenyi et al., 2002). Aβ4-8 (Phe-Arg-His-Asp-Ser, FRHDS) is a possible ligand for integrin-like receptors (Lorenzo et al., 2000; Sabo et al., 1995) and we presume that this Aβ fragment and its analogues might serve as inhibitors for the Aβ-integrin interactions. On the basis of previous results, novel compounds of four pentapeptides (analogues of Aβ4–8, 17–21, 30–34, and 38–42) were synthetized. One of them (Phe-Arg-His-Asp-Ser amide, FRHDSa) is an integrin ligand analogue; Leu-Pro-Tyr-Phe-Asp amide (LPYFDa; (Datki et al., 2004) is a slightly modified analogue of Soto's Leu-Pro-Phe-Phe-Asp (LPFFD). Arg-Ile-Ile-Gly-Leu amide (RIIGLa) and Arg-Val-Val-Ile-Ala amide (RVVIAa) are water-soluble analogues of the peptides that we earlier prepared and studied (propionyl-Ile-Ile-Gly-Leu amide and Gly-Val-Val-Ile-Ala amide). In order to differentiate among the possible protective mechanisms, two kinds of ejection patterns were used. The pentapeptides were either co-iontophorised, or applied in mixture form with Aβ1-42. Parallel with the *in vivo* studies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and *in vitro* electrophysiological experiments were performed, however, only the *in vivo* studies are in the focus of the present thesis.

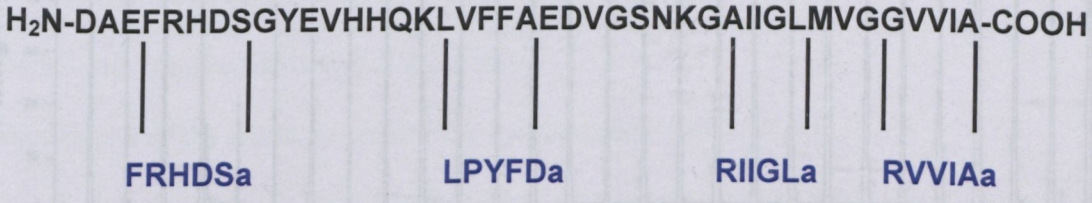

Fig. 11.: Sequence of Aβ1-42 and the derived pentapeptides. FRHDSa (Aβ4-8), LPYFDa (an analogue of Aβ17-21), RIIGLa (an Arg substitute of Aβ30-34) and RVVIA (an Arg substitute of Aβ38-42) were tested against Aβ1-42 induced enhancement in vivo.

Co-iontophoresis of Aβ1–42 and the individual pentapeptides

When the individual pentapeptides were administered together with Aβ1–42 (co-iontophorized), the neuromodulatory effect of Aβ1–42 was decreased. FRHDSa (n = 8) and LPYFDa (n = 8) prevented the enhancement of the NMDA-induced responses caused by Aβ1–42, although a small increase could be observed after 15 min: the response maxima were 150 ± 28% and 157 ± 37% (Aβ1–42 response maximum was 267 ± 19%). Application of RIIGLa (n = 4), however did not significantly prevent the Aβ1– 42 induced enhancement of the responses: 30 min after ejection, the responses did not differ from those induced by Aβ1–42. RVVIAa co-iontophoresis (n = 7) offered no prevention from the Aβ1–42 effect either. However, the responses after RVVIAa administration were enhanced up to a maximum of 314 ± 48%, which was reached between 22 and 40 min after ejection. The individual pentapeptides alone did not change the NMDA-evoked firing rate (data not shown).

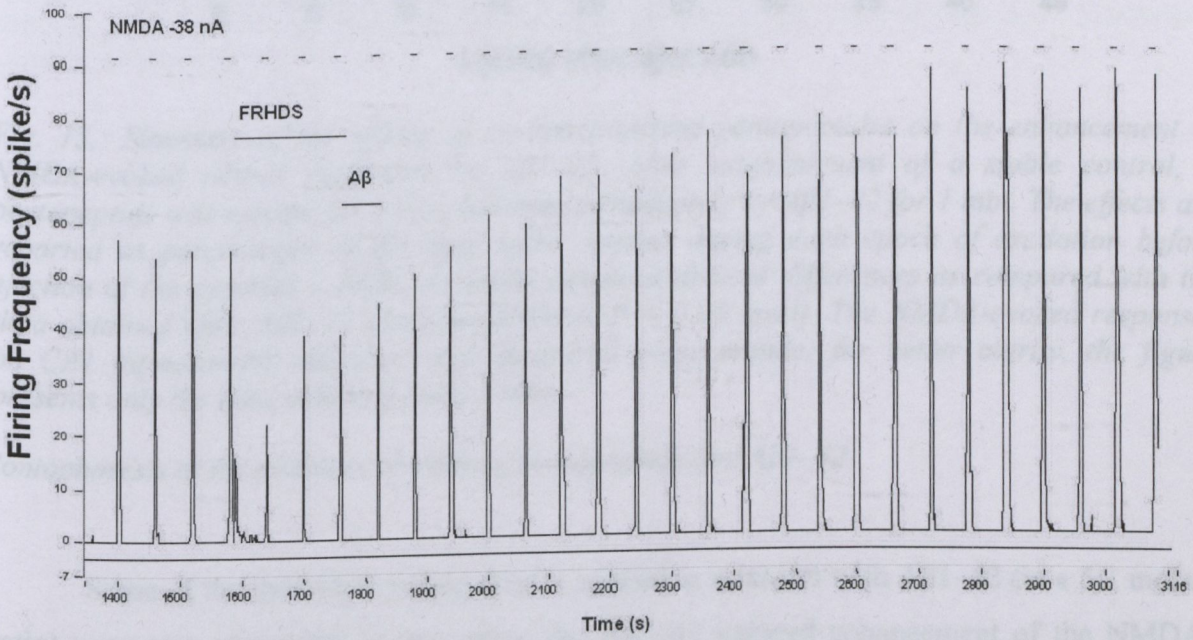

Fig. 12.: A representative peristimulus histogram of a co-iontophoretic application of the pentapeptide FRHDS and Aβ1-42. The CA1 neuron was excited by the repetitive ejection of NMDA (ejected at – 38 nA). FRHDS was ejected for 3 min at +100 nA, while Aβ1-42 for 1 min at -0.5 μA. Excitation occurred every min.

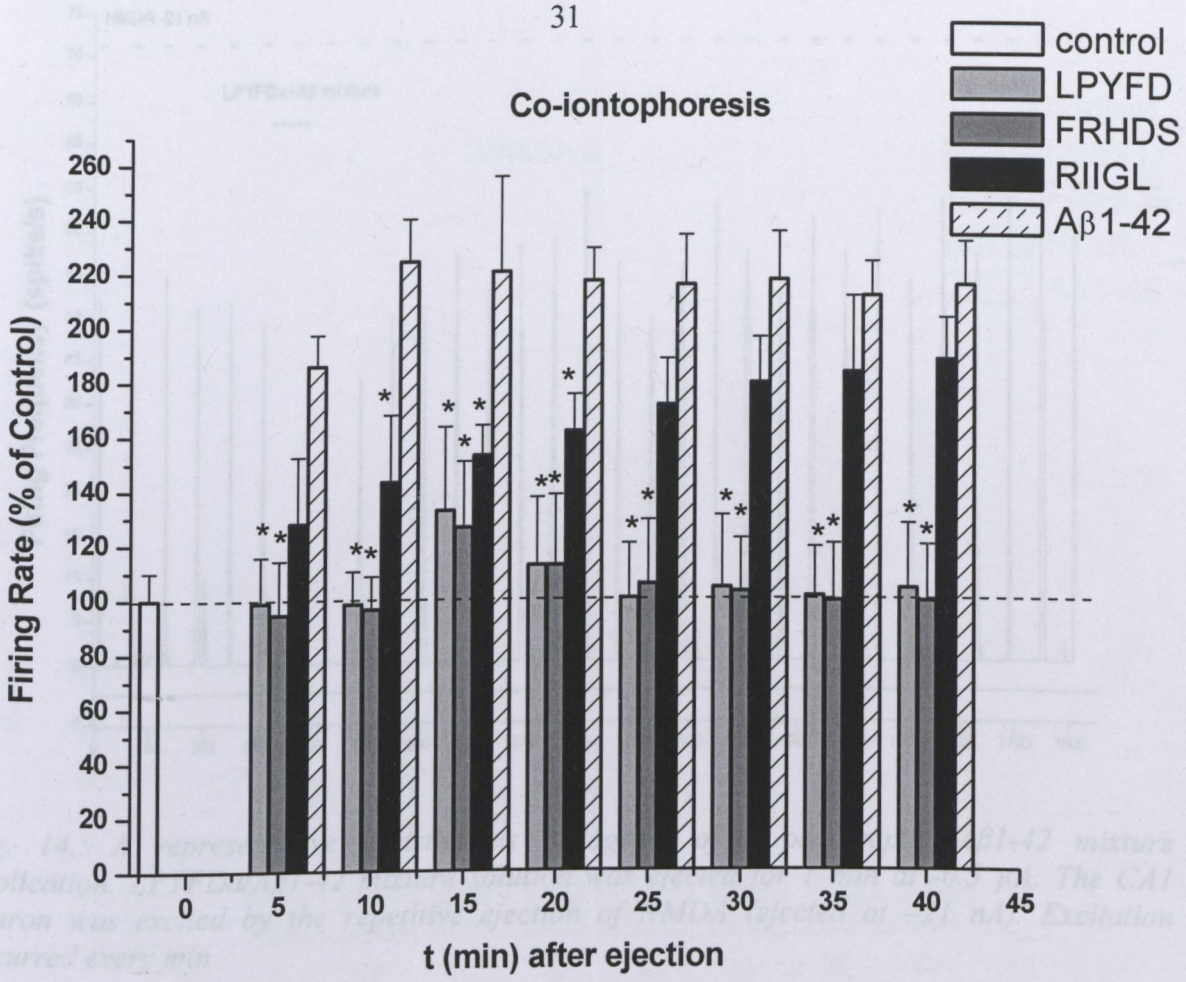

Fig. 13.: Summary of the effects of co-iontophorized pentapeptides on the enhancement of NMDA-evoked neural responses by Aβ1–42. After establishment of a stable control, a pentapeptide was ejected for 3 min, followed immediately by Aβ1–42 for 1 min. The effects are reported as percentages of the total spike number during each epoch of excitation before ejection of the peptides ± SEM. Asterisks denote statistical differences as compared with the data obtained after Aβ1–42 ejection (ANOVA, $P < 0.05$ level). The NMDA-evoked responses on CA1 hippocampal neurons were measured every minute; for better clarity, the figure presents only the data obtained every 5 min.

Iontophoresis of the mixtures containing pentapeptide and Aβ1–42

Some of the individual pentapeptides applied in mixtures with Aβ1–42 (in a 5:1 molar ratio) were also successful in preventing the Aβ1–42 induced enhancement of the NMDA induced responses. FRHDSa proved to be inactive in these experiments: no significant difference was observed between the enhancing effect of Aβ1–42 and the mixture of Aβ1–42 and FRHDSa after 25 min (n = 13). The LPYFDa-containing mixture did not result in an NMDA-evoked response enhancement; this peptide fully prevented the neuromodulatory effect of Aβ1–42 (n = 6). RIIGLa demonstrates a partial counteraction against Aβ1–42: the maximum response was 174 ± 24% (n = 7). Similarly to the results of the coiontophoresis, RVVIAa was ineffective also in the mixture form (n = 6): the maximum firing rate was larger (317 ± 51%) than that induced by Aβ1–42 (267 ± 19%) between 17 and 25 min after ejection.

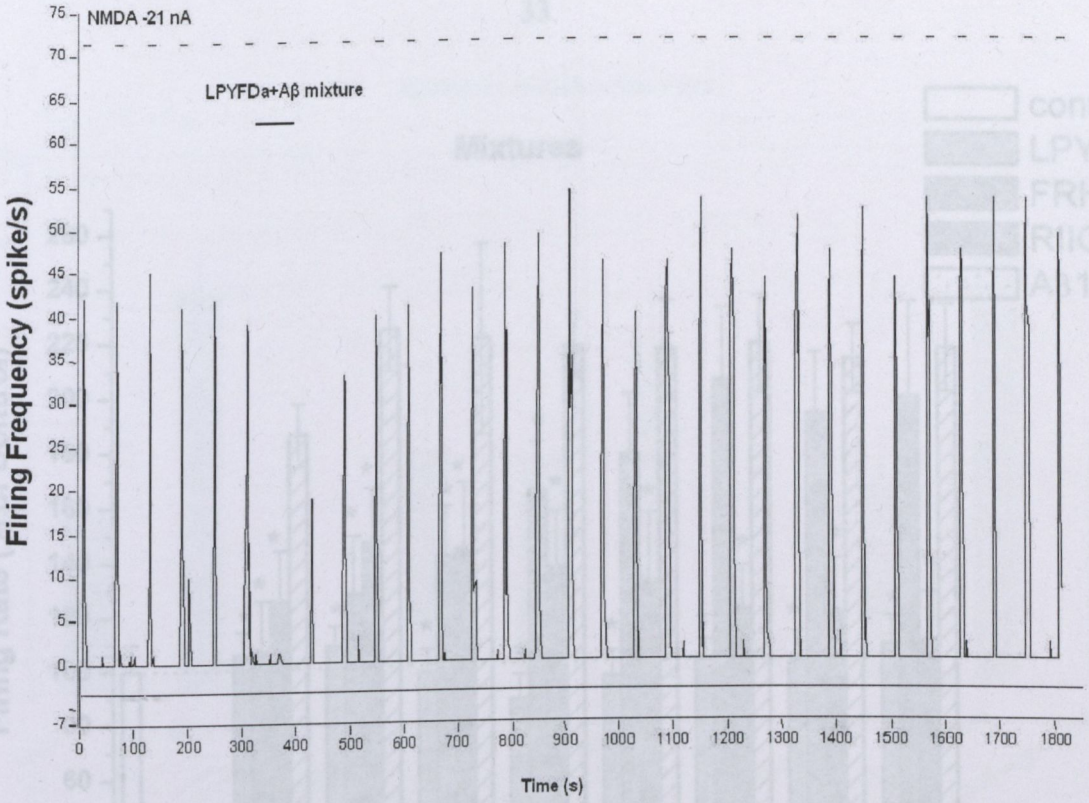

Fig. 14.: A representative peristimulus histogram of a pentapeptide/Aβ1-42 mixture application. LPYFDa/Aβ1-42 mixture solution was ejected for 1 min at -0.5 μA. The CA1 neuron was excited by the repetitive ejection of NMDA (ejected at –21 nA). Excitation occurred every min

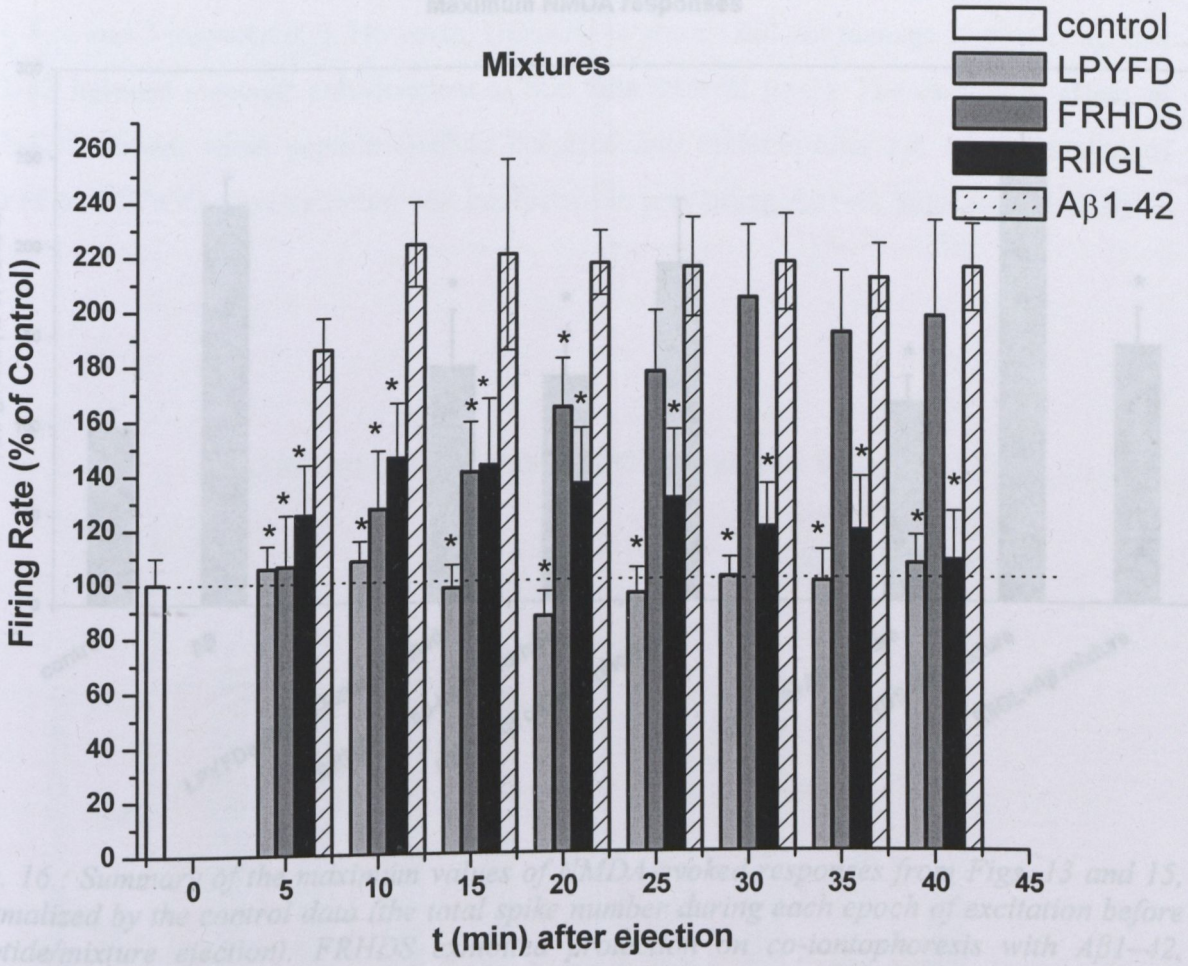

Fig. 15.: Summary of the effects of mixtures containing a pentapeptide and Aβ1–42 and of Aβ1–42 alone on the NMDA-evoked responses. The effects are reported as percentages of the total spike number during each epoch of excitation before ejection of the mixtures or Aβ1–42 (±SEM). Asterisks denote statistical differences as compared with the data obtained after peptide ejection (ANOVA, $P < 0.05$ level). The NMDA-evoked responses on CA1 hippocampal neurons were measured every minute; for better clarity, the figure presents only data obtained every 5 min.

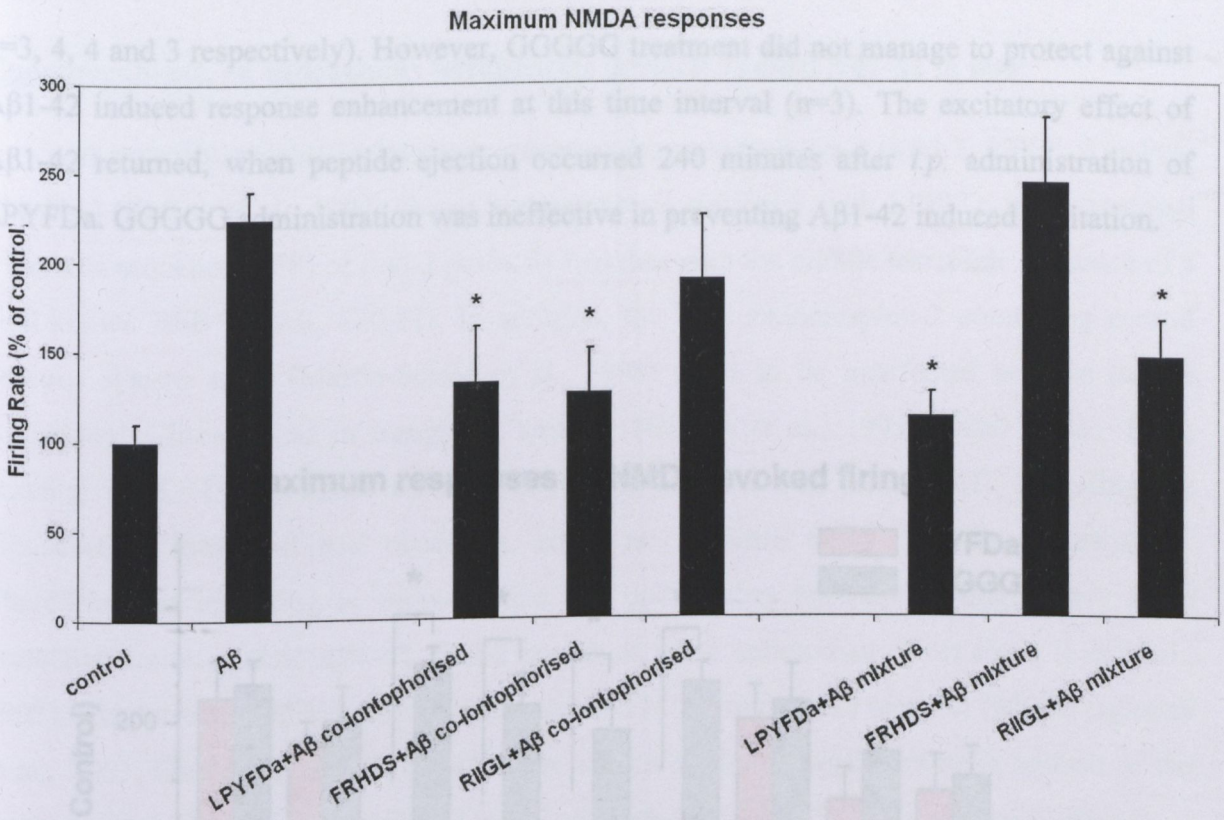

Fig. 16.: Summary of the maximum values of NMDA-evoked responses from Figs. 13 and 15, normalized by the control data (the total spike number during each epoch of excitation before peptide/mixture ejection). FRHDS exhibited protection on co-iontophoresis with Aβ1–42, RIIGL was effective in the mixture form, and LPYFDa proved to be protective in both application methods. Asterisks indicate significant differences as compared with the Aβ1–42-induced enhancement (Student's t test $P < 0.05$).

Intraperitoneal administration of LPYFDa

Our next aim was to elucidate, whether LPYFDa, the most effective protective pentapeptide, is capable of preventing Aβ1-42 induced effects when administered intraperitoneally (*i.p.*). A control pentapeptide, pentaglycin (GGGGG) was used as control.

First, we tried to find a stable single-unit activity within the CA1 region. After reaching a stable control sequence for NMDA induced firing, we ejected Aβ1-42, just as described previously. At the same time, LPYFDa was injected intraperitoneally. Since one recording session lasted for about 35 minutes, and activity was recorded from 4 rats, we were not able to standardize the recording time points. Instead, we pooled the data from every 40 minutes after *i.p.* administration.

All neurons recorded 0 min or 40 min after *i.p.* administration of the peptides showed a clear Aβ1-42 induced NMDA-response enhancement. In contrast, the NMDA-induced firing frequency of cells of LPYFDa treated animals did not rise after Aβ1-42 ejection between 80 and 200 minutes after *i.p.* administration (137 ± 11%, 120 ±14%, 120 ± 12%, 125 ± 11%;

n=3, 4, 4 and 3 respectively). However, GGGGG treatment did not manage to protect against Aβ1-42 induced response enhancement at this time interval (n=3). The excitatory effect of Aβ1-42 returned, when peptide ejection occurred 240 minutes after *i.p.* administration of LPYFDa. GGGGG administration was ineffective in preventing Aβ1-42 induced excitation.

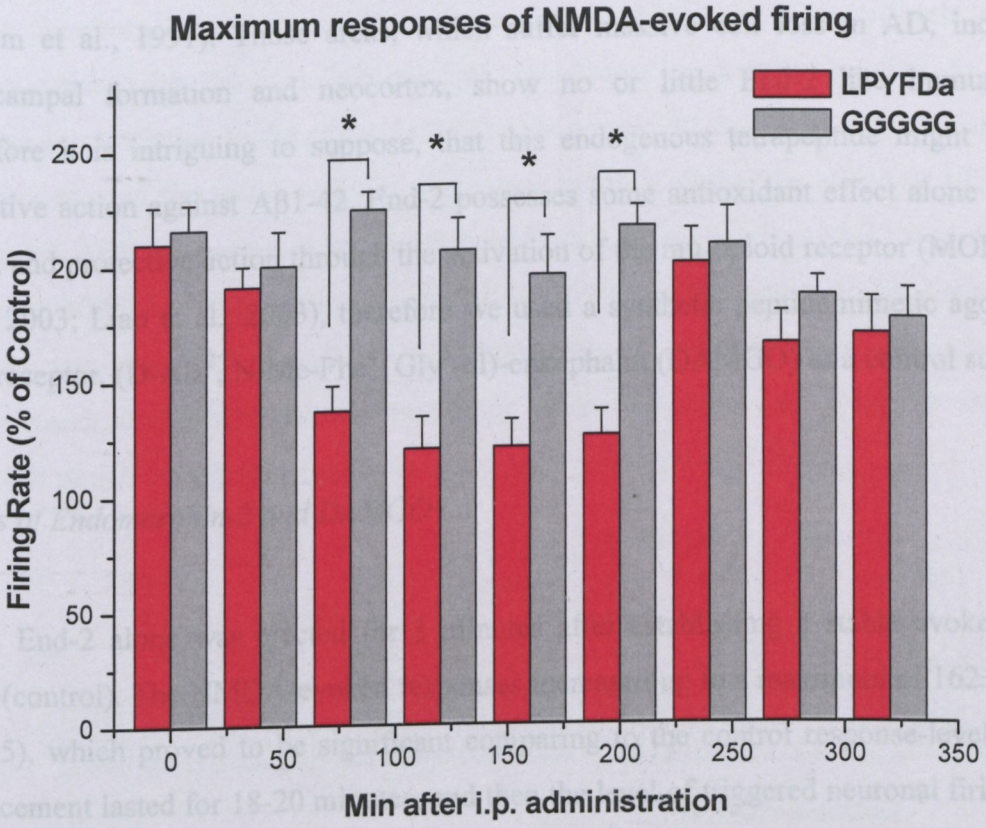

Fig. 17.: Summary of the maximum values of NMDA-evoked responses, normalized by the control data (the total spike number during each epoch of excitation before Aβ1-42). LPYFDa protected against the NMDA-response enhancement effect of Aβ1-42 between 80 and 200 min after i.p. administration of the pentapeptide. The control pentapeptide, pentaglycin was not able to interfere with Aβ1-42 induced excitation. Asterisks indicate significant differences (Student's t test $P < 0.05$).

Endomorphin-2, an endogenous tetrapeptide protects against Aβ1-42 *in vivo*

A recently isolated endogenous tetrapeptide, endomorphin-2 (End-2, YPFF, Tyr-Pro-Phe-Phe) (Zadina et al., 1997) shows high structural similarity with LPFFD: the C-terminal tripeptide sequence (PFF) of End-2 perfectly matches with the middle tripeptide sequence of a well-known BSB peptide, LPFFD. In addition, the high endomorphin-2 containing central nervous system areas (Martin-Schild et al., 1999) seem to be unaffected both in human Alzheimer's disease and in transgenic animals (Games et al., 1995; Hsiao et al., 1996; Joachim et al., 1991). Those areas, which suffer massive cell loss in AD, including the hippocampal formation and neocortex, show no or little End-2 like immunreactivity. Therefore it is intriguing to suppose, that this endogenous tetrapeptide might have some protective action against Aβ1-42. End-2 possesses some antioxidant effect alone (Lin et al., 2003), and protective action through the activation of the mu-opioid receptor (MOR) (Iglesias et al., 2003; Liao et al., 2003), therefore we used a synthetic peptidomimetic agonist of the same receptor, (D-Ala^2, N-Me-Phe^4, Gly^5-ol)-enkephalin (DAMGO) as a control substance.

Effects of Endomorphin-2 and DAMGO

End-2 alone was ejected for 3 minutes after establishing a stable evoked neuronal firing (control). The NMDA-evoked responses increased up to a maximum of 162±14% (n=9, $P<0.05$), which proved to be significant comparing to the control response-level. Response enhancement lasted for 18-20 minutes, and then the level of triggered neuronal firing returned to control level. DAMGO application for 3 minutes resulted in similar significant response enhancement to 167±5% (n=5, $P<0.05$). However, the evoked responses did not return to the control level within the time frame of the experiment, the enhancement proved to be long lasting. Application of the vehicle had no effect on the rate of evoked neuronal firing (n=6, data not shown).

Effect of mixtures containing Aβ1-42 and End-2 or Aβ1-42 and DAMGO

The peptide mixtures were administered iontophoretically for one minute at -0.5μA. In that way, only the negatively charged Aβ1-42 was ejected, End-2/DAMGO could not leave the micropipette, unless absorbed on the surface of different Aβ1-42 assemblies. Application of the End-2 and Aβ1-42 mixture did not result in enhancement of the NMDA responses: the

level of neuronal firing remained at 115±21% (n=6). In contrast, ejection of the DAMGO and Aβ1-42 containing solution enhanced NMDA-evoked firing. The maximum response to 269±37% remained, and it did not return to control level (n=6, $P<0.05$).

Co-iontophoresis of Aβ1-42 and End-2 or Aβ1-42 and DAMGO

In order to elucidate the protective mechanism of End-2 against the effect of Aβ1-42, co-iontophoretic administration was used. End-2 and DAMGO were ejected right before Aβ1-42 application with the same current and time frame as described above, to ensure the effective concentration in the close vicinity of the neuron. In that way, μ-opioid receptors will be activated, and the drugs will not exert their putative protective effect on the surface of aggregated Aβ1-42 species. After ejection of End-2, immediately Aβ1-42 was applied. The maximum NMDA-evoked response reached 348±65%, and the enhancement remained during the measurements (n=7, $P<0.05$). Co-iontophoresis of DAMGO with Aβ1-42 resulted in similar response enhancement. The maximum evoked response was 238±37%, and the effect did not diminish during the time frame of the experiment (n=6, $P<0.05$).

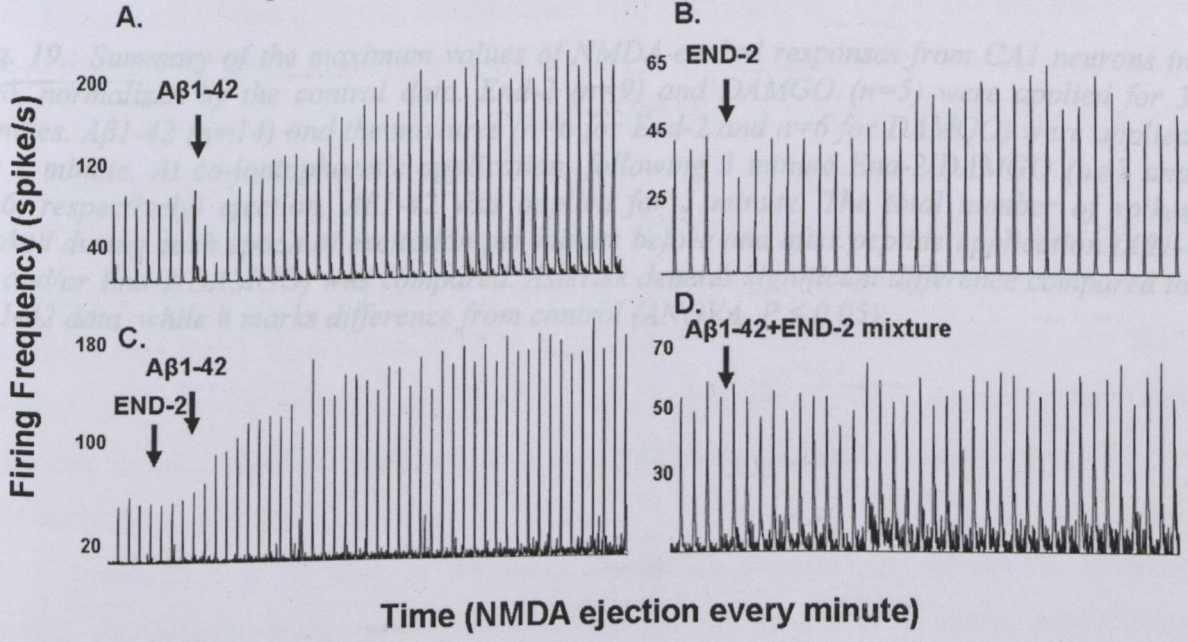

Fig.. 18.: Peristimulus histograms representing the effect of Aβ1-42 (A), endomorphin-2 (B), endomorphin-2 and Aβ1-42 co-iontophoresis (C) and endomorphin-2 and Aβ1-42 containing mixture (D) on the NMDA-induced neuronal firing recorded from CA1 hippocampal neurons in vivo. Arrows denote the time of peptide application.

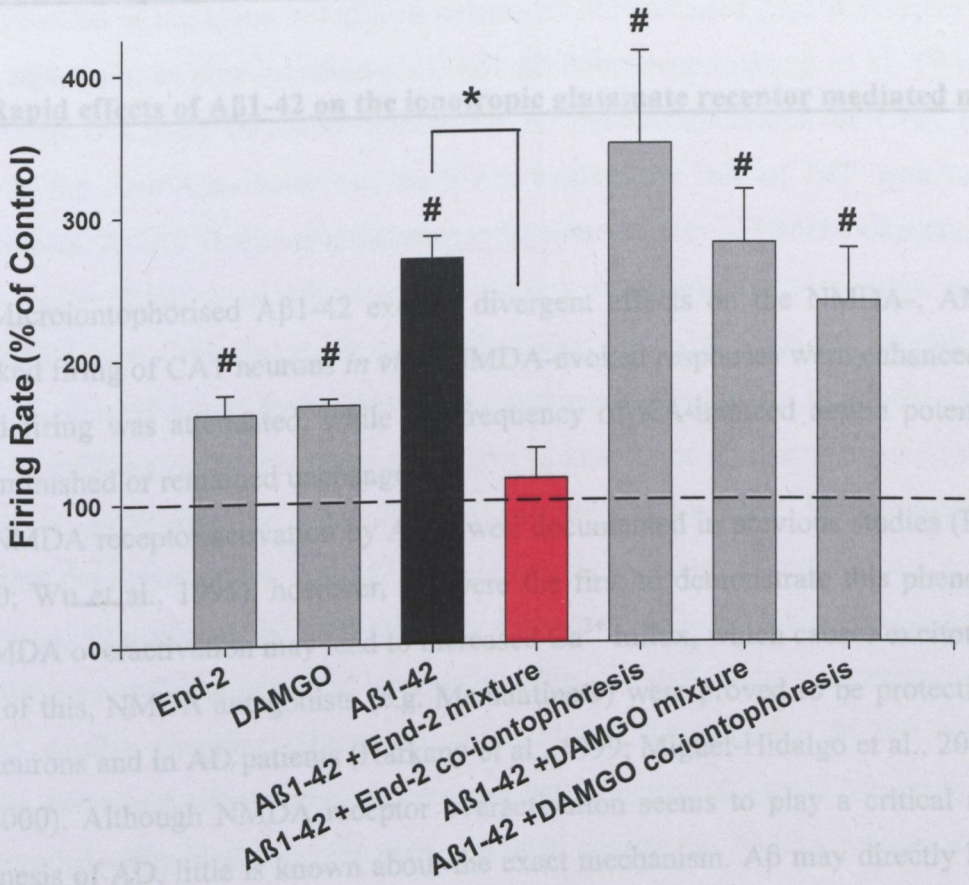

Fig. 19.: Summary of the maximum values of NMDA-evoked responses from CA1 neurons in vivo, normalized by the control data. End-2 (n=9) and DAMGO (n=5) were applied for 3 minutes. Aβ1-42 (n=14) and the mixtures (n=6 for End-2 and n=6 for DAMGO) were applied for 1 minute. At co-iontophoretic application, following 3 minute End-2/DAMGO (n=7 and n=6, respectively) ejection, Aβ1-42 was applied for 1 minute. The total number of spikes evoked during each epoch of excitation per minute before and after peptide application (Aβ1–42 and/or End-2/DAMGO) was compared. Asterisk denotes significant difference compared to Aβ1-42 data, while # marks difference from control (ANOVA, $P \leq 0.05$).

Discussion

Rapid effects of Aβ1-42 on the ionotropic glutamate receptor mediated neuronal firing

Microiontophorised Aβ1-42 exerted divergent effects on the NMDA-, AMPA- and KA-evoked firing of CA1 neurons *in vivo*. NMDA-evoked responses were enhanced, AMPA-triggered firing was attenuated, while the frequency of KA-induced action potentials were either diminished or remained unchanged.

NMDA receptor activation by Aβ is well documented in previous studies (Harkany et al., 2000; Wu et al., 1995), however, we were the first to demonstrate this phenomenon *in vivo*. NMDA overactivation may lead to increased Ca^{2+} influx, which causes excitotoxicity. In support of this, NMDA antagonists (e.g. Memantine®) were proved to be protective against Aβ on neurons and in AD patients (Harkany et al., 1999; Miguel-Hidalgo et al., 2002; Ruther et al., 2000). Although NMDA receptor overactivation seems to play a critical role in the pathogenesis of AD, little is known about the exact mechanism. Aβ may directly bind to the NMDA receptor complex, as was demonstrated by (Cowburn et al., 1997), and this interaction might increase the single-channel conductivity or the opening probability. Another possibility is that Aβ1-42 triggers a yet unknown cascade by activating an extracellular protein, which may result in the alternation of NMDA receptor-subunit phosphorilation level (Sze et al., 2001). The latter seems more plausible, because a pentapeptide, which is supposed to bind to the integrin receptors, is capable of protecting against Aβ1-42 induced NMDA receptor excitation (see later). In addition, NMDA mediated firing-enhancement emerged only 8-12 minutes after Aβ1-42 ejection, while compounds, acting directly on a receptor must exert their effects more rapidly. Therefore, a direct modulation of NMDA receptor complex by Aβ1-42 is quiet unlikely.

Since there is no data in the literature, which suggest Aβ1-42–AMPA receptor binding, diminishing of the AMPA-mediated signals may be due to an altered intracellular regulatory mechanism. A crucial step in the induction of NMDA-independent long term potentiation (LTP) of synaptic plasticity is the trafficking of AMPA receptors into the postsynaptic site, increasing the concentration of glutamate-responsive elements directly at the neuron-neuron junction (Kakegawa et al., 2004; Kolleker et al., 2003). As these mechanisms are closely related to the intact cytoskeleton, and given the wealth of data indicating Aβ1-42 interference with the normal cytoskeletal working (e.g. (Datki et al., 2004; Gamblin et al., 2003)), such altered mechanisms cannot be ruled out. Alternatively, the

changed function of the C-jun N-terminal kinases (JNK)-mediated AMPA receptor recycling pathway represents another possibility of Aβ1-42 interference. Wang et al. (Wang et al., 2002a) found that JNK inhibitors rescue the Aβ-induced inhibition of LTP. Further, a decrease in the AMPA-mediated responses can explain the lack of LTP seen in Aβ1-42-treated animals. AMPA receptors are known to be essential for the NMDA-dependent LTP in the CA1 region. GluR-A (a subunit of the AMPA receptor) knock-out adult rats do not develop LTP in the CA1 (Jensen et al., 2003). Moreover, LTP could not be elicited in the presence of an AMPA antagonist in *in vitro* experiments (Debray et al., 1997). A synaptic function impairment emerges before the physical integration of the synapses (Yao et al., 2003) and Aβ pathology (Ye et al., 2004) during the onset of AD. As a consequence, we propose that the hypofunction of AMPA, and possibly the altered working of KA receptors, contributes significantly to the LTP blockade caused by Aβ1-42. It has been suggested that the selective neuronal loss in the CA1 region in AD is due to the selective expression of Ca^{2+}-permeable AMPA channels (Blanchard et al., 2004). Our results do not support this hypothesis, because AMPA-mediated responses had disappeared almost completely from all of the recorded cells. However, the cells remained active: the NMDA responses increased after Aβ1-42 application. The discrepancy between the two results may be explained; Blanchard et al. utilized an *in vitro* Ca^{2+} influx measurement, where they specifically blocked all AMPA receptors on cultured neurons. In this way, antagonising of AMPA receptors may cause a complete blockade in the working of NMDA receptors, because the blocking Mg^{2+} ions remain in the NMDA ion channels (see (Freitas da Rocha et al., 2001) for review). In contrast, under our *in vivo* circumstances, the modulating effects of the ejected Aβ1-42 emerges only at the near vicinity of the electrode that do not include the entire membrane surface of the CA1 neuron; each of the ejected compounds (NMDA, AMPA, KA, Aβ1-42) reach only a limited surface of the observed cell. Therefore, due to the constant "synaptic bombardment", the membrane potential of the neuron may reach the threshold, where Mg2+ ions exit from the NMDA ion channels allowing NMDA-evoked neuronal firing.

Our results reveal that the changes in the NMDA- and AMPA-evoked responses do not follow the same temporal pattern. We used a mixture of mature Aβ1-42 fibrils and protofibrils, as revealed by transmission electron microscopy (TEM) images (Szegedi et al., 2005b). One study (Ye et al., 2004) suggests that the modulation of NMDA and non-NMDA glutamate receptors by Aβ1-42 is dependent on the aggregation state. Protofibrils enhance NMDA receptors, while fibrils act on non-NMDA receptors. However, both aggregation states increased the neuronal excitability in patch-clamp experiments and the different

aggregation states of Aβ may be responsible for the differences in temporal change. Further studies are needed to elucidate the exact mechanisms of this finding.

The effects of Aβ1-42 on the KA-induced firing distinguished two groups of cells. In the first group, Aβ did not change the KA responses. However, the NMDA responses enhanced, and Aβ therefore acted on the neurons. In the other group, Aβ sharply decreased the KA-evoked responses, similarly to the AMPA-elicited firing. KA receptors are localized presynaptically and postsynaptically in the CA1 region, and it is therefore difficult to fully explain the above found results. Additionally, KA in a relatively high concentration has been reported to act on AMPA receptors. To make the situation even more complicated, recent papers report on the possible metabotropic effects of KA receptors (Rodriguez-Moreno and Lerma, 1998) (Frerking et al., 2001).

Aβ attenuates the field excitatory postsynaptic potential (fEPSP) in the hippocampal region and in the primary motor cortex both *in vitro* and *in vivo* (Stephan et al., 2001; Szegedi et al., 2005a). Our results show that, despite the activation seen in the NMDA-evoked firing, the AMPA- and even KA-induced responses massively decreased. The sum of these changes in the working of fast-acting excitatory ionotropic channels points to a net decrease in the neuronal excitability and synaptic activity, which is presumed to be measured by fEPSP induction.

Aβ1-42 derived pentapeptides protect neurons against Aβ1-42

The protective effects of 5 selected pentapeptides derived from Aβ1–42 towards the neuromodulatory effects of Aβ1–42 were studied.

LPYFDa proved to be protective against iontophoresed Aβ1–42. It prevented both the NMDA-evoked firing enhancement, and AMPA responses attenuation. This peptide, an analogue of a well-known BSB (LPFFD), can be bound with high affinity to the Aβ1–42 monomer (Hetenyi et al., 2002). Therefore, we hypothesize that a rapid and strong binding occurs between the pentapeptide and Aβ1-42 assemblies, preventing further interaction with the cells. Accordingly, LPYFDa was protective both on co-iontophoretic administration and by ejection in a mixture with Aβ1–42.

The arginin substitute of C-terminal fragment of Aβ1-42, **RVVIA** exhibited some protection against Aβ1–42 toxicity in the MTT assay (Hetenyi et al., 2002; Szegedi et al., 2005a), this effect was not significant, and the same peptide was found to be inactive against microiontophorised Aβ *in vivo*.

RIIGL was also protective in MTT assay and in *in vitro* electrophysiological studies (Szegedi et al., 2005a). This peptide displayed protective action only in the mixture form (after preincubation with Aβ1–42) and proved to be inactive when simultaneously ejected (co-iontophoresis) in *in vivo* electrophysiological studies. We assume that the pentapeptide binds to Aβ, however the time of contact between Aβ1–42 and RIIGL is relatively short in the co-iontophoretic experiments. The pentapeptide probably cannot cover the surface of Aβ assemblies, owing to the short contact. During preincubation, the pentapeptide molecules have enough time to bind to the surface of Aβ1–42, neutralizing its neuromodulatory effects.

FRHDS, a protective agent in MTT assay, was effective in *in vivo* electrophysiological experiments only when ejected by co-iontophoresis to the cells; its preincubation with Aβ1–42 (mixture form) did not provide protective action. This difference in effects may not serve as an unquestionable evidence of different protective mechanism, but may indicate a different site of action. Literature data reveal that Arg-His-Asp-Ser (RHDS) and similar peptides bind to the integrin-like receptors of the cell surface (Ghiso et al., 1992; Sabo et al., 1995; Saporito-Irwin and Van Nostrand, 1995). We presume that Phe-Arg-His-Asp-Ser acts as a competitive inhibitor by binding to the integrins of the cellular membrane, inhibiting further interaction of the integrin like receptors with the Aβ1–42 aggregates. Since the inhibition of this interaction may prevent the neuromodulatory effects of Aβ1-42, further investigation and experiments may yield a novel therapeutic direction in the treatment of AD.

We conclude from our results that **LPYFDa** and **RIIGL** protect neurons from the modulatory effect of Aβ1–42 by binding to Aβ. **FRHDS** probably acts rather as integrin-type receptor-binding ligand. **RVVIA** proved to be practically inactive against the neuromodulatory effects of Aβ1–42.

Intraperitoneally administered LPYFDa protects against Aβ1-42 induced neuronal excitation

Intraperitoneally administered LPYFDa was able to defend against Aβ1-42 induced neuronal excitation. The temporal window of effectiveness was relatively wide (between 80 and 200 min after administration), since the compound was not optimized for enzyme resistance and blood-brain barrier permeability. The emerging point of the neuroprotective effect of the administrated compound is in good correlation with other studies, which described similar intraperitoneal central nervous system (CNS) targeting peptide administration (Li et al., 2006; Tohyama et al., 2004; Wang et al., 2002b). Pentaglycin, the control peptide did not display protection.

An endogenous pentapeptide, endomorphin-2 protects neurons against Aβ1-42

The amyloid induced enhancements of NMDA-evoked responses were also diminished by End-2. This protective effect was not mediated through the activation of mu-opioid receptor (MOR), because DAMGO, a synthetic MOR agonist was not able to attenuate the Aβ1-42 mediated effect. Results of the two kinds of *in vivo* iontophoretic experiments ("mixture" and "co-iontophoresis") provided additional evidence, that the activation of mu-opioid receptors does not yield protection against Aβ1-42. Co-iontophoretic application of Aβ1-42 with either End-2 or DAMGO resulted in NMDA-induced response-enhancement, similar to the effect of β-amyloid. However, when Aβ1-42 was preincubated with End-2, no β-amyloid induced enhancement could be measured, similarly with LPYFDa and RIIGL. In co-iontophoretic experiments, the ejection of MOR agonists preceded Aβ1-42 application in order to activate the μ-receptors. In contrast, at application of the mixture solutions, the pH values and the ejection polarities were chosen in that way, that only Aβ1-42 and the possibly bound End-2 and DAMGO could leave the capillary. Under these conditions presumably no significant MOR activation occurred due to the ejection of the mixtures. Since the sequence of End-2 resembles to LPFFD and LPYFDa, a similar protective mechanism is assumed. The tetrapeptide binds to the surface of aggregated Aβ1-42, as revealed by radioligand binding studies and circular dichronism experiments (Szegedi et al., 2006), however, it does not behave as a BSB compound. According to transmission electron microscopy and quasi-light scattering studies, End-2 does not interfere with the aggregation of Aβ1-42 (Szegedi et al., 2006). Therefore, the most plausible way of protection is the surface coverage of Aβ1-42 by the tetrapeptide, preventing further detrimental interaction of the formed complex with the cells.

Being an endogenous substance, End-2 might play a role in the actual AD progression, and possibly assent to the area selective cell loss seen in the disease and transgenic animals. Central nervous system areas, with high End-2 content were found to be unaffected by Aβ induced damage (Games et al., 1995; Hsiao et al., 1996; Joachim et al., 1991). Those areas, which suffer cell loss in AD, including the hippocampal formation and neocortex, show no or little End-2 like immunreactivity. Only a few endogenous molecules have been reported or suggested to have protective effect against β-amyloid, *i.e.* taurine (Louzada et al., 2004) and kynurenic acid (Savvateeva et al., 1999), which target the excitotoxic imbalance either by γ-aminobutyric acid $_A$ ($GABA_A$) receptor activation, or by NMDA receptor inhibition. Shifting the net level of neuronal excitability towards inhibition attenuates Ca^{2+} entry. Decreased intracellular Ca^{2+} level provides less probability of apoptotic cascade initiation. Another

endogenous compound, melatonin has a more complex neuroprotective mechanism. In addition to its antioxidant properties, it also binds to $GABA_A$ receptor (Louzada et al., 2004). Additionally, melatonin blocks fibrillogenesis of Aβ1-42 *in vitro* (Pappolla et al., 1998). Endomorphin-2 may join to this list of neuroprotective endogenous compounds, however it has a more distinct and direct neuroprotective action against Aβ1-42, namely it blocks the harmful amyloid-cell interaction.

The effect of mu-opioid receptor activation on the NMDA evoked neuronal firing indicated that the action potentials of pyramidal cells were recorded. The distribution of MOR in the hippocampus displays cell type specificity: only the GABA-ergic interneurons express this type of inhibitory, metabotropic receptor (Drake and Milner, 1999; Drake and Milner, 2002). Therefore, excitation after End-2 and DAMGO ejection indicates a disinhibitory mechanism (McQuiston and Saggau, 2003): μ-receptor bearing interneurons might be inhibited by agonists, resulting in an attenuation of the inhibitory input to the pyramidal cells, increasing their excitability has increased.

Perspectives for future drug development for AD treatment

Current drugs against AD treatment furnish only symptomatic treatment and have their limitations. Compounds targeting Aβ directly offer protection from the majority of β-amyloid-induced neuromodulatory effects and may serve as leads for further drug development. Our most promising agent, LPYFDa is capable of penetrating the blood-brain barrier and protect, when administered intraperitoneally. However, the pentapeptide, may be optimized for having longer defensive period, therefore it may serve as a lead compound for future drug development.

Our other promising compound is End-2, which, as being a tetrapeptide, may have better bioavailability and more favorable pharmacokinetics than the pentapeptides. Since it is an endogenous substance, one may speculate to increase its level in the organism would be beneficial for patients suffering from AD. However, approaches aimed to increase End-2 level in the brain presumably would not offer rational treatment of AD patients. The activation of μ-opioid receptors increases the net excitability in the CA1 region, and may result in focal epileptic seizures (McQuiston and Saggau, 2003). Consequently, in the treatment of Alzheimer's disease, the sequence of End-2 is what may be exploited as a lead sequence for development of peptidomimetic drugs that will not activate μ-opioid receptors.

Summary

First, we have demonstrated a rapid deleterious effect of aggregated Aβ1-42 on the normal excitatory synaptic function. Ejected Aβ1-42 enhanced NMDA-induced neuronal firing, while AMPA evoked responses were diminished. KA receptor mediated firing remained unchanged, or attenuated. These results may explain the fEPSP decreasing and LTP attenuating effect of Aβ1-42. In addition, these phenomena might form the basis of the cognitive decline in early AD, where neuron loss is low. In the light of these results, the usage of drugs acting on AMPA receptors may be feasible in that stage of the disease.

Second, we have tested 4 pentapeptides derived from Aβ1-42 against Aβ induced neuronal excitation. By using two patterns of peptide ejection, we have shown different putative protective mechanisms of these pentapeptides. RIIGL and LPYFD were effective in the mixture form with Aβ1-42, indicating a direct binding between the two compounds. In contrast, FRHDS displayed a competitive inhibitor-like effect, preventing the interaction of Aβ1-42 with the cells.

Third, we have proved that *i.p.* administration of the most potent neuroprotective pentapeptide, LPYFDa may offer protection against Aβ1-42 in a relatively moderate temporal period. By optimizing the structure of this pentapeptide, a more effectiveness could be reached.

Fourth, we have shown that an endogenous tetrapeptide, End-2 defends against Aβ1-42 elicited NMDA response enhancement. Since the tetrapeptide was only effective in the mixture form with Aβ1-42 and DAMGO did not display protection, End-2 defends in a MOR independent manner.

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